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PREFACE

In the present volume, the first to be issued under our joint editorship, we have continued the policy of earlier volumes, of choosing a few subjects from the diverse fields of work in which physical or physicochemical ideas and methods are being applied to biological problems. In this volume we are able, for the first time, to include articles on the use of isotopes to investigate metabolic processes. Other subjects discussed in detail are: the electric organs of fishes, the thermodynamics of agglutination of red cells, muscle structure and function, photosynthesis, and the structure of bone.

Considering the variety of the topics which come within our field, there has been, with each volume, a temptation to try to recast and limit the definition of what should be covered under the heading of "Biophysics and Biophysical Chemistry." We have had a good many discussions on this point and have come to the conclusion that, as biophysics is a many-sided discipline, no useful purpose is served by excluding matters which appear to be almost solely of physicochemical or physiological interest at the present time.

We shall continue, therefore, to apply a liberal interpretation to the limits of our field and try to maintain a reasonable balance between its various aspects. Many subjects, perhaps predominantly biochemical, are clearly of importance to "physiological" biophysicists, as well as to physicists and physical chemists working with biological materials, and this will be increasingly the case as the study of physiological mechanisms approaches the molecular level. However, we do not wish to enter very far into the biochemical field and propose to confine such articles to topics which are closely related to the main lines of biophysical interest, or in which biophysical methods of investigation have been extensively applied.

It has also been suggested that the selection of subjects in any one volume should be restricted to one particular aspect of the field. However, we have come to the conclusion that this would make more acute the problems of segregation and of timing and would probably lead to an undesirable degree of specialization of the individual articles. After all, what really matters to the editors is the timeliness of any particular subject coming up for review and the willingness of a competent author to write it. These are in practice more important limitations than any which the editors would impose by dissecting the wide field which they attempt to cover.

Thanks are again due to our contributors for fulfilling their assignments in good time and for their readiness to comply with our suggestions as to the lay-out of their articles.

J. A. V. Butler
B. Katz

London, 3 December, 1956

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THE MECHANISMS OF DISCHARGE OF THE ELECTRIC ORGANS IN RELATION TO GENERAL AND COMPARATIVE ELECTROPHYSIOLOGY

Harry Grundfest

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1

THE MECHANISMS OF DISCHARGE OF THE ELECTRIC ORGANS IN RELATION TO GENERAL AND COMPARATIVE ELECTROPHYSIOLOGY

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I. Introduction

In that part of his *Commentary* which is entitled "Conjectures and some conclusions" Galvani (1791) wrote, "This one thing seems particularly proper and peculiar of the *Torpedo* and cognate animals that at their will and pleasure they can direct electricity outside the skin, and expel it so that it completes its circulation outside the body, and with . . . quantity and force. . . . But perhaps in animals of this class this indicates more abundant quantity and force, not really a different nature; and perhaps some time, devices can be found whereby effects of this sort can be obtained in other animals also." This conjecture, shared by many others, as well as a more general curiosity about their "peculiar force," impelled many studies of electric fishes. Another of Galvani's conjectures was proved as correct as the first: ". . . electric circulations of this sort, discovered and described by us in other animals, not only their strength and relations but also their ways and instrument, perhaps will be able to shed some light on the same circulation in the *Torpedo* and cognate animals." A long series of electrophysiological investigations of nerve and muscle did eventually serve to explain the discharge of the electric fishes (Bernstein, 1912; Keynes and Martins-Ferreira, 1953; Altamirano *et al.*, 1953 and 1955a), in terms of a common mechanism of electrogenic action (Bernstein, 1902; Overton, 1902; Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952).

Nevertheless, another of Galvani's conjectures also proved right, if one disregards his primitive idea of an electric fluid between muscle and nerve: ". . . from more diligent discussion and observation of these animal (i.e. electric) organs which are fitted for this function, these forms will be able to receive light. The instruments (i.e. mechanisms) perhaps will be similar, and the terminals of the electric circuit, namely muscles and nerves, the same." Recent studies of innervated preparations of electric organs studied either as groups of cells (Altamirano *et al.*, 1953) or as individual unit complexes (Altamirano *et al.*, 1955a; Brock *et al.*, 1953b) have disclosed new information. In

correlation with other work on electric organs (e.g. ALBE-FESSARD *et al.*, 1951; ALBE-FESSARD and CHAGAS, 1954, 1955a,b; CHAGAS and ALBE-FESSARD, 1954); on the nerve-muscle complex (FATT and KATZ, 1951; DEL CASTILLO and KATZ, 1954b); and on central nervous synapses (e.g. BROCK *et al.*, 1952, 1953a; COOMBS *et al.*, 1955b,c,d; ECCLES *et al.*, 1954), they have provided a general view of the mode of synaptic transmission which will form the framework of this chapter. Comparative data on other electrogenic systems will be included briefly in this account of the discharge of electric organs[1] to illustrate the essential similarity of properties and mechanisms of these various excitable tissues. Some interpretations and predictions (GRUNDFEST, 1956a,b,c), several already confirmed by new findings (KAO and GRUNDFEST, 1955, 1956; PURPURA and GRUNDFEST, 1956a,c; GRUNDFEST and PURPURA, 1956) will be indicated.

The point of departure is a fundamental differentiation of postjunctional excitable membrane (i.e. that which is associated in synaptic action with a nerve supply to produce an electrical response), and nonjunctional excitable membrane. While neurohumoral excitation of the former is widely admitted, evidence (some of which will be presented) indicates that, in all cases thus far accessible to study, the postjunctional (or postsynaptic) membrane differs fundamentally from the nonjunctional in that it is not excitable by applied electrical stimulation nor by local circuit depolarization (cf. GRUNDFEST, 1956a). Therefore, transmission of synaptic action is obligatorily through neurohumoral excitation of this membrane. Also, the postsynaptic membrane is not at all, or relatively little, affected by the subsequent electrical changes caused by the spikes of the nonsynaptic component, or by fields generated by activity of other cells.

Furthermore, whereas the electrically excitable membrane is capable of electrogenic processes which result in either graded or all-or-nothing responses, the postsynaptic has different electrogenic processes always capable of responding gradedly. The eel electroplaque is endowed with both electrically and synaptically excitable types of membrane, the electrical properties of each, however, being distinct. The electrically excitable membrane of the electroplaque also reveals, and more easily than do the similar membranes of other cells, under appropriate conditions, entirely graded or all-or-nothing responsiveness. Although comparable studies of other electric organs are not yet available, the evidence at hand indicates that the best known of these probably possess only synaptically activated membranes which are not electrically excitable.

In summary, this review attempts to evaluate the electrophysiology

[1] I am indebted to Dr. R. D. KEYNES for a manuscript copy of his succinct account of electric fishes prior to its publication (1956).

of the fish electric organs in the contexts of general and comparative electrophysiology. However, the reader must be warned that the approach is more radical than is generally accepted. A recent review of chemical transmission, by a leading proponent of that view, opened with the remark: "In considering the mechanism of central and sensory transmission we are faced with the problem that, whatever our bias, we cannot state with certainty whether the transmission is chemical or electrical" (FELDBERG, 1954, p. 85). Nevertheless, it is the present writer's view that considerable data have already become available to show the existence of two major classes of electrogenic mechanisms. One, residing in electrically excitable membrane, appears to be adapted to conductile activity and is found in tissues predominantly of this function—axons, and muscle fibres. The other resides in membrane which is not electrically excitable, but which responds electrogenically to specific adequate stimuli—chemical, mechanical, photic, or thermal. It appears functionally in cells or regions of cells in which the electrogenesis is graded with the intensity of an external stimulus and is associated with sense organs or junctions between cells. In most of the tissues this electrically inexcitable, electrogenic membrane is closely associated with conductile, electrically excitable membrane, and the electrogenesis of the former is capable of initiating responses in the latter. The evidence for this general hypothesis will be indicated below.

II. HISTORICAL[2]

The peculiar ability of electric fishes to produce sensations unpleasant or painful to a person who holds them was known to antiquity in the

[2] General references will be given here to the older work, and detailed citations of that literature omitted: (i) DU BOIS-REYMOND (1881), gathered the material of his gifted student CARL SACHS, whom he had sent in 1876 to study *Electrophorus* on the site in Venezuela where HUMBOLDT had reported in 1799 the "battle between horses and eels" (cf. DU BOIS-REYMOND, 1881, p. 88). Working under field conditions SACHS made a number of important discoveries, such as: the large-celled organ which now bears his name; the electric organ of *Eigenmannia* (*Sternopygus*) *virescens*; and the differences between direct and indirect excitability of the eel electric organ (facilitation, fatiguability and susceptibility to curare of the neurally evoked response). The latter observations, confirmed in modern days (ALBE-FESSARD *et al.*, 1951a,b; CHAGAS and ALBE-FESSARD, 1954; ALTAMIRANO *et al.*, 1953, 1955a,b) puzzled older workers who had gathered most of their data on the *Torpedines*, the skates, and *Malapterurus*. The notes which SACHS left behind on his death at 25, in a mountaineering accident, were amplified by his famous teacher who included historical and comparative introductions to each phase of the work, and summarizing interpretations. Two appendixes by FRITSCH give a summary of the latter's anatomical studies. (ii) Although BIEDERMANN never worked with electric fishes, the chapter on these in his classic *Elektrophysiologie* (1895) is an admirable summary, chiefly based on DU BOIS-REYMOND, but also including newer data. (iii) GOTCH (1900) gave an account in English. (iv) GARTEN (1910), (v) BERNSTEIN (1912) and (vi) ROSENBERG (1928), in publications which are readily available, summarized the progress of the field, with copious references. Other aspects will be found in CRANE (1950) and KEYNES (1956). The anatomy of the organs is discussed in the

exemplars of the Mediterranean *Torpedines* and the Nile catfish, *Malapterurus electricus*. Their power to repel aggressors or to stun prey was also known. An Arab physician, ABD-ALLATIF (cf. BALLOWITZ, 1899, p. 6) described with remarkable accuracy the effects produced on himself when touching a discharging *Malapterurus*. He reported that fishermen also felt these effects on handling only the net containing the fish, effects which disappeared when the fish died. The Jesuit Father, GODIGNO, in the seventeenth century, noted that a live *Malapterurus* thrown amongst a lot of recently dead fish caused the latter to move, as though brought back to life. This may be regarded as probably the first, although unconscious, example of electrophysiological experiments. Yet the anatomical basis of these properties was not sought until the investigations of FRANCESCO REDI in 1666. He described in *Torpedo* two crescent-shaped bodies which he inferred to be of muscular origin and in which the force of the effects seemed to be concentrated. The nature of this force was elegantly, logically, but incorrectly specified by BORELLI (1685), an important pioneer in the development of mechanics. He supposed that these organs, as muscles, contracted at high rates, the blows resulting from these when the animal was touched giving rise to unpleasant sensations as does a blow on the elbow. This explanation seemed adequate to HALLER, LINNAEUS and RÉAMUR, as late as 1750, since knowledge of forces other than mechanical was absent. However, only a few years after the discovery of the Leyden jar a Dutch administrator-naturalist (s'GRAVESANDE) and a French botanist (ADANSON) suggested that the organs acted as generators of electric discharges. WILLIAMSON (1773), WALSH (1775) and CAVENDISH (1776) demonstrated that in many of their properties the discharges behaved like electricity, and that an electric discharge could mimic the stimulating effects of the organ. JOHN HUNTER (1775) gave a thorough anatomical "account of the *Gymnotus electricus*." GIRARDI published in 1786 anatomical work on the "organi ellettrici della Torpedine." SPALLANZANI, using the electrophore, showed that during a discharge the dorsum of *Torpedo* becomes positive relative to its ventral surface. VOLTA, on developing his "pile," proposed that it be called an artificial electric organ.[3] Never-

above and also by BALLOWITZ (1938), and by COUCEIRO and AKERMAN (1948). BALLOWITZ (1899) lists 299 references on electric fishes published up to that time.

[3] VOLTA apparently had his tongue in cheek placing his electric machine on a par with the electric organ. It will be recalled that VOLTA correctly disputed GALVANI's claim to have demonstrated a flow of electricity pre-existing between nerve and muscle and set into motion by touching the two with a metallic (or bimetallic) arc. VOLTA showed that GALVANI was dealing with electricity generated at the metallic contacts with the electrolytes and stimulating the tissues. He was even the first to observe the different excitatory effects of cathode and anode, in 1791. VOLTA's chief aim from then on was to prove that the electricity was generated in nonliving systems. Despite GALVANI's later demonstration of the rheoscopic preparation in which excitation took

theless DAVY, in 1828, questioned the bioelectric nature of the organ's activity, and FARADAY (1844), applying the powerful new physical tools to the study of the most powerful of the electric organs—that of the South American electric eel—could not completely satisfy his eight criteria of action describing electrical phenomena. In a communication to FARADAY, who remained unconvinced, DU BOIS-REYMOND (1881, p. 160) pointed out that the different electrical circuit properties of Galvanic batteries, Leyden jar arrays, and electric organs, could account for their different effects. It was not until DU BOIS-REYMOND (1848) showed that nerve and muscle were truly electrogenic, that the bioelectric nature of the discharge was accepted. The last half of the nineteenth century then brought forth a rich harvest of anatomical and physiological data by many workers.

III. STRUCTURE OF ELECTRIC ORGANS

III. 1. *Electroplaques*

Practically all our knowledge of the embryology and morphology of the electric organs derives from the second half of the nineteenth century (cf. GOTCH, 1900; GARTEN, 1910; ROSENBERG, 1928). The organs are composed of ordered complexes of individual cells (Fig. 1), each rather richly supplied with nerve fibres. The latter make contact with only one of the external surfaces of the electroplaques (or electroplates) (Fig. 2), but in some forms (e.g. *Torpedo, Raia*) the innervation is more dense than in others (e.g. *Electrophorus*). The electroplaques of all known forms except *Malapterurus* derive from muscle fibres, but in that fish they probably originate from secretory cells in the skin. The structure of the individual cells is essentially similar in all forms (Fig. 3). The electroplaque is located in a compartment, the protoplasmic portion of which occupies sometimes only part, but sometimes most of the space. The remainder is filled with a rather transparent, gelatinous material, blood vessels and nerve fibres. The innervated face of the electroplaque usually is digitated in structure, but not nearly as much as the rest. Some cytological differentiations exist between the innervated and noninnervated faces of the cells, but the significance of these differences is obscure.[4]

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place without metals, by what we now know as the demarcation current of bioelectric activity, VOLTA's incomparably greater scientific acumen and rigour won the day. The demonstration of an electric generator composed of metals and salts therefore appeared to be the final triumph in VOLTA's controversy with GALVANI. Although VOLTA never satisfactorily explained in terms of his "pile" the origin of electric discharges of fish, the study of animal electricity, including that of the electric fishes, nevertheless lagged until DU BOIS-REYMOND gave electrophysiology a scientific methodology and interesting data.

[4] Electron-microscopic work in this laboratory (P. BERNSTEIN, unpublished) has failed to disclose conspicuous differences between the membranes of the eel electroplaque at these different surfaces, despite the profound functional differences which will

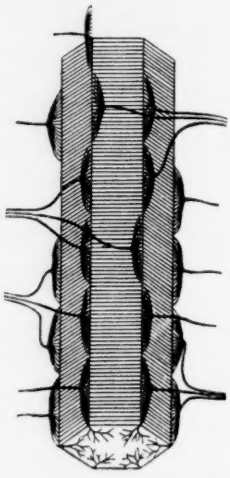

Fig. 1. A prism of electroplaques from *Torpedo*. The organ is so tightly packed that the cells assume a roughly hexagonal shape. Each electroplaque is innervated at its ventral surface by six nerve bundles which enter the cell at the corners. The arrangement of the organ and its innervation are shown in Fig. 4. (From BIEDERMANN, 1895, after FRITSCH.)

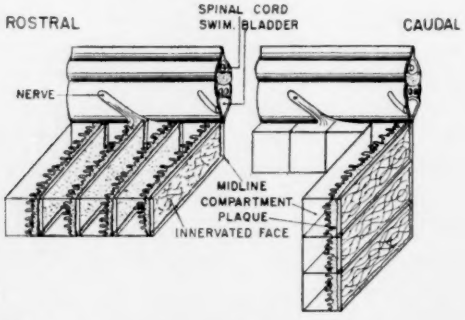

Fig. 2. Diagram of the arrangement of electroplaques in *Electrophorus*. The caudal faces of the cells are innervated from segmental nerve trunks. At least 3 trunks supply each cell of the Sachs organ (as in Fig. 22). On the left is shown a row of cells arrayed for series summation of voltages. On the right are shown 3 cells of a column. These discharge in parallel. (From ALTAMIRANO *et al.*, 1953.)

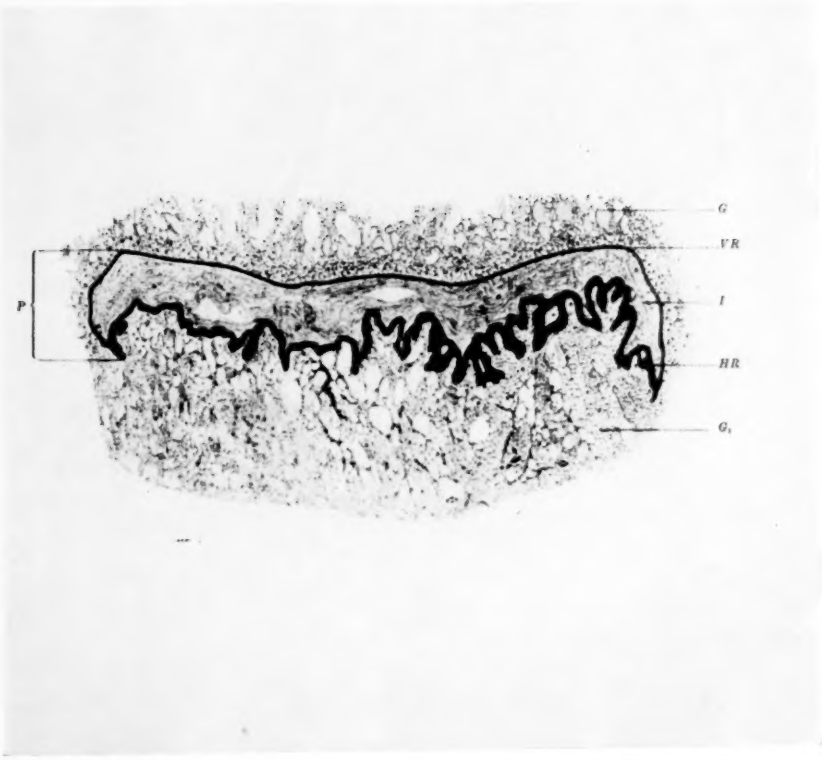

Fig. 3. An electroplaque from *Raia*. The cell proper P is surrounded by a gelatinous mass G. This electroplaque is innervated at its anterior surface VR. The caudal surface HR is highly digitated. The interior I has some fine structure, but this is as yet poorly understood. (From ROSENBERG, 1928, after BALLOWITZ.)

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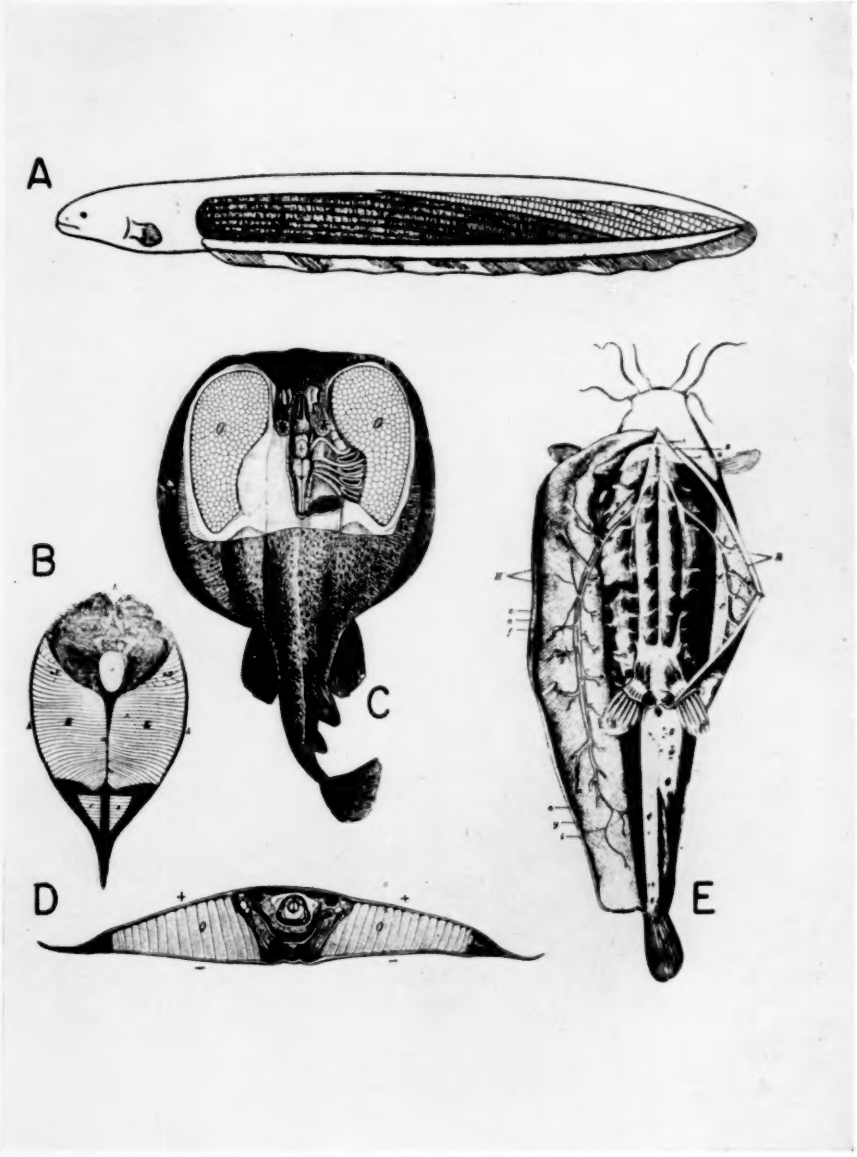

Fig. 4. Arrangement of electric organs in *Electrophorus* (*A*, *B*), *Torpedo* (*C*, *D*) and *Malapterurus* (*E*). In the eel, the electric organs occupy most of the length of the fish. The main organ (shaded in *A*, marked *E* in *B*) is the largest. The Sachs organ (clear rectangles in *A*, marked *nE* in *B*) begins above the main organ, and its large cells comprise the caudal portion. The Hunter organ lies below, separated from the rest by connective and fatty tissue. In *Torpedo* the bilateral organ (*O*) is composed of stacks of electroplaques packed tightly together. Their dorsoventral orientation makes the upper surface of the fish positive during discharge. The innervation of the right side is shown in *C*, arising from one of the two electric lobes of the brain. The electric organ of *Malapterurus* (*O*) lies within the skin (*H*). The single axon innervating all the cells of one half the fish runs along with the blood vessels to the skin. (From various sources.)

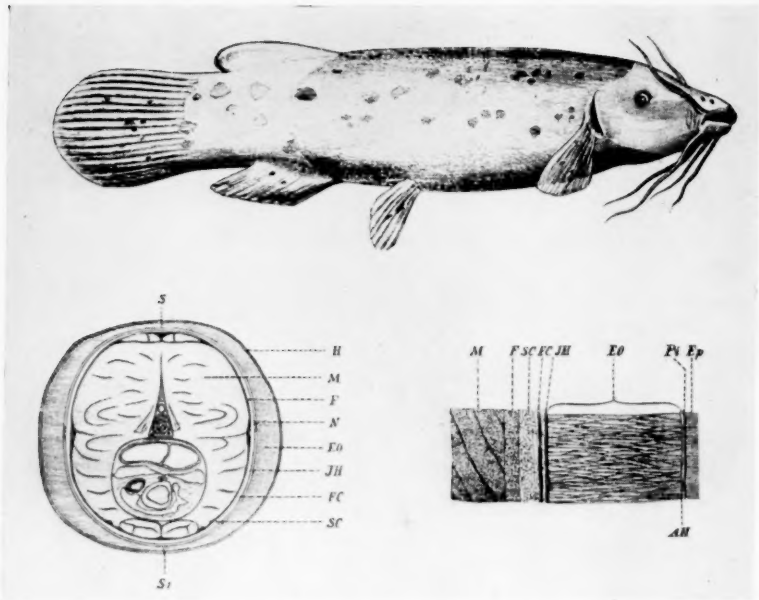

Fig. 5. The electric organ of *Malapterurus*. The shaded anterior portion of the fish shows the location of its electric organ. Below, *left*, is a diagrammatic cross-section of the fish showing the organ (*EO*) lying within the skin (*H*), separated from the muscle (*M*) by various layers of connective tissue. *S* and S_1 are septa separating the organs of the two sides. *Right*: The thin electroplaques which comprise the electric organ are seen in coronal section. (From BALLOWITZ, 1899.)

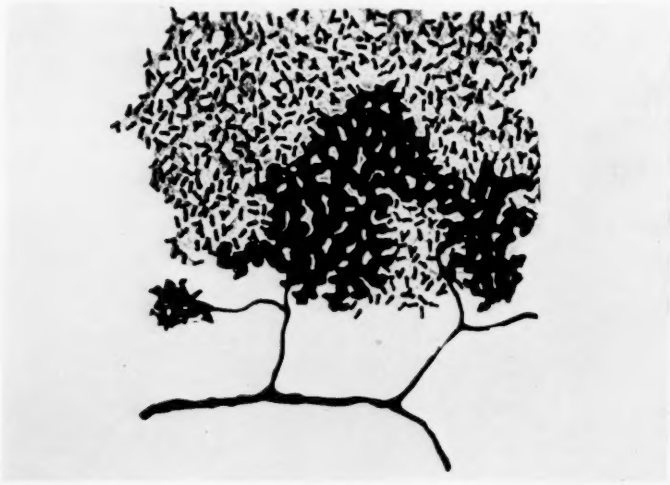

Fig. 6. Innervation of a *Torpedo* electroplaque. The terminal arborizations and thickenings of the nerve fibre shown, and of others, appear to occupy most of the innervated face of the cell, which is the ventral in this fish. (From ROSENBERG, 1928, after BALLOWITZ.)

III. 2. *Organization*

The gross structure of the organs into which the electroplaques are assembled differs in the various fishes. In *Torpedo* the cells are stacked vertically like a column of coins. These dorso-ventral columns (Fig. 4), replacing muscle of the non-electrical relatives of the fishes, are packed so closely that their cells assume roughly hexagonal shapes. In *Electrophorus* and *Raia* the electroplaques are essentially rectangular, with their long axis more or less at right angles to the spinal cord (Fig. 2). The arrays of these cells, stacked both dorso-ventrally and rostro-caudally, are agglomerated in *Electrophorus* into three well-defined bilateral organs, occupying most of the length of the fish (Fig. 4). The linear density of the electroplaque population is highest rostrally, declining from a maximum of about 10/mm in the main organ to about 1/mm or less in the caudal portion of the hindmost organ (that of SACHS). The other fishes, with the exception of *Malapterurus*,[5] conform more or less closely to one or the other of these patterns. In the latter named (Fig. 5) roughly lozenge-shaped compartments lie packed within the thickened skin. Discs within these compartments are probably the electroplaques. Almost 5 millions of these, arrayed in about 3000 stacks of 1600 each, are said to comprise this organ.

III. 3. *Innervation and central control mechanisms*

The innervation of the electric organs also varies with the different fishes. *Malapterurus* has two giant cells, bilaterally located in the medulla. The single axon of each is said to innervate all the electroplaques of one side, dividing in the process so much that the sum of the areas of the chief compartmental branches alone is about 350,000 times the area of the fibre when it leaves the cell.[6] In *Torpedo*, four nerve

be described below. COUTEAUX (1955) mentions unpublished electron-microscopic work on *Torpedo* electroplaques. Cholinesterase is largely concentrated at the innervated surface of the eel electroplaque (COUCEIRO *et al.*, 1955a). Preliminary anatomical studies of *Sternarchus albifrons* (COUCEIRO *et al.*, 1955b) indicate electric organs composed of cylinders (electrotubes) in which the electrogenic structures form rings spaced along the length. However, another of the continuously emitting Gymnotids, *Eigenmannia virescens*, has long, narrow compartments, rostro-caudally oriented with their protoplasmic portions confined to the posterior ends (unpublished data). SACHS (cf. DU BOIS-REYMOND, 1881, p. 69) described these long cells, although not their detailed structure. Joined as they are end to end, these cells might give the appearance of tubes.

[5] BALLOWITZ (1899) considers that *Malopterurus* is more correct etymologically. This spelling is frequently found in older literature. The exceptional origin of the electric organ of this fish and, as will be noted frequently below, the exceptions in some of its properties, make it all the more regrettable that work on this form almost completely stopped with the last century, despite its importance, which BALLOWITZ (1899) pointed out.

[6] The possible implications of this extreme branching for theories of excitation and propagation in nerve may be far reaching, but have not been studied. The axon, although only about 8–10 μ in diameter, conducts at about 40 m.p.s. at 12°C (GOTCH, 1900).

trunks emerge from each of the pair of electrical lobes behind the cerebellum (Fig. 4). Their distribution to the electroplaques of a column is shown in Fig. 1, and an appreciation of the density of their synaptic contacts with the innervated surface of the electroplaque may be derived from Fig. 6. In *Electrophorus*, however, several hundred nerve trunks emerge bilaterally from the spinal cord, their cells of origin being distributed along the length of the latter. The efferent cells controlling the discharge in all electric fishes are rather large (COUCEIRO and FESSARD, 1953), though not as big as those of *Malapterurus* (200 μ). The efferent neurons of the spinal cord of the eel are controlled in turn by a large-celled nuclear mass in the medulla.[7]

IV. PHYSIOLOGICAL CHARACTERISTICS OF THE RESPONSES IN DIFFERENT ELECTRIC ORGANS

IV. 1. *Polarization of the discharge*

PACINI, about 1853, formulated the rule that the discharge of electric fishes is so directed as to make the innervated surface of the electroplaque negative. This holds true for all the fishes except *Malapterurus* (Fig. 7). In the *Torpedines* the ventral surfaces of the dorso-ventrally stacked cells are innervated, and the dorsum of the fish becomes positive. The innervation is of the dorsal surfaces in *Astroscopus* and the ventral surface becomes positive. In some of the *Gymnotids*, including *Electrophorus*, and in the *Mormyrids*, the innervated faces are caudal, the head end becoming positive during the discharge. The innervation of *Raia* electroplaques is the reverse of this, the caudal end becoming positive. Only in *Malapterurus* is PACINI's rule broken. The electroplaques are innervated at their caudal surfaces, but the tail end is also positive during the discharge.

IV. 2. *Voltage on discharge*[8]

The maximum voltages of the discharges when the different species of fishes are stimulated also vary. The fresh water *Electrophorus* develops

[7] Some of the properties of nervous-control mechanisms in *Electrophorus* have been studied by ALBE-FESSARD (1954), with MARTINS-FERREIRA (1951, 1953) and with CHAGAS and COUCEIRO (1954) and also by COUCEIRO and FESSARD (1953, 1955). Multiple innervation of the same electroplaque of the Sachs organ was described by ALTAMIRANO *et al.* (1953) ALBE-FESSARD has also studied the central control mechanisms in *Torpedo* (1951b, 1952) with BUSER (1953) and with SZABO (1954). The latter (SZABO, 1955) has demonstrated a bulbar nucleus in *Raia* which initiates reflex activity. Both in *Raia* and in *Electrophorus* higher cerebral levels seem to exert inhibitory actions on the nuclei of central command since sections of the brain stem in both just rostral to the command nucleus causes enhanced excitability (COUCEIRO and FESSARD, 1955; SZABO, 1955).

[8] The literature is replete with references to different discharge voltages in small or large fish of the same species (cf. EILENFELD, 1926; ROSENBERG, 1928, p. 878). It seems to be well established (delle Chiaie-Babuchin rule, cf. DU BOIS-REYMOND, 1881,

the highest voltage (maximal recorded 866 V, cf. EILENFELD, 1926; ROSENBERG, 1928, p. 878), but in other *Gymnotids* the discharge is less than 1 volt (COATES *et al.*, 1954). The fresh water *Malapterurus* and the

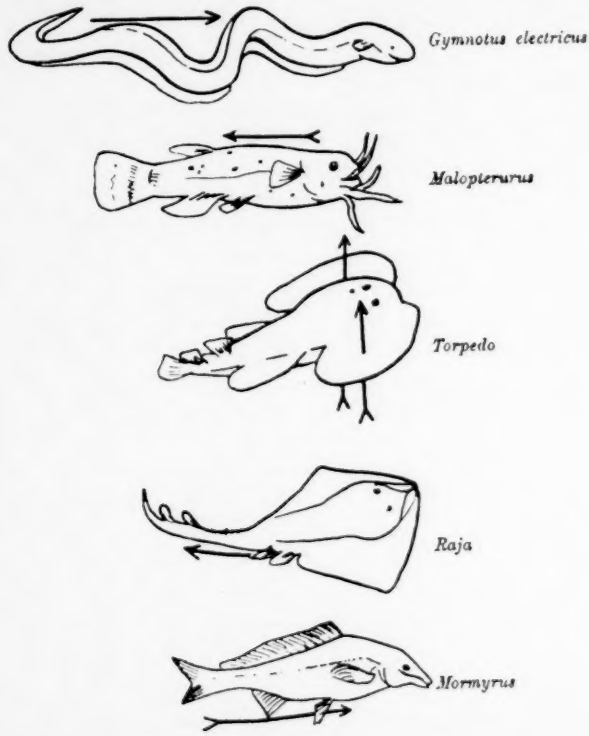

Fig. 7. The directional flow of current in different electric fishes. The arrows give the flow in the animal. Their heads therefore indicate the positive pole as measured externally. (From GARTEN, 1910.)

p. 31) that the cells of the electric organ are preformed and that their number remains constant during growth of the animal. Unless this is found wrong, the e.m.f. of the organs might be expected to remain constant, provided all their cells are always functional. Smaller recorded voltages would then have to be explained by greater relative short-circuiting of the generated e.m.f. by the body and external fluid. On the other hand, it is also possible that in younger fish some or many of the electroplaques are not functionally innervated. Another difficulty in interpreting the voltages recorded during the discharges of the fish stems from their apparent deterioration. This was not observed for *Electrophorus*, the electroplaques of fish which were kept in the laboratory for more than one year giving the same responses as those of the recently caught (ALTAMIRANO, 1955; ALTAMIRANO, *et al.*, 1955a), but it may be a more prominent factor in other forms (cf. ROSENBERG, 1928, p. 879), perhaps operating through a failure of innervation. On the other hand, ELLIS (1913) showed that the *Gymnotids* are capable of regenerating lost portions of the body behind the anal region. This might suggest that the delle Chiaie-Babuchin rule needs re-examination.

sea dwelling *T. occidentalis* develop maximal discharges recorded as about 350 V and 220 V respectively. Other *Torpedines* (*T. marmorata* and *Narcine brasilensis*) have yielded only some 40 to 50 volts, as has the teleost *Astroscopus*. *Raia* and the *Mormyrids* as well as the aforementioned *Gymnotids* have only weak discharges (cf., KEYNES, 1956).

The voltage is a function of the number of electroplaques stacked in a column. In *Electrophorus* the variation of linear density of the electroplaque array has permitted a direct correlation between the number of cells per unit length and the voltage developed in the discharge of that length of the organ (COX *et al.*, 1945).

IV. 3. *Number and frequency of discharges in the reflex response*

The number of the naturally evoked discharges also varies. Particularly those fishes capable of strong discharges are electrically silent except when stimulated. Then, the activity comprises a brief train (Fig. 8) at frequencies which vary, but may reach about 200/sec. (cf. FESSARD, 1947). This reflexly evoked response is subject to fatigue after some hundreds or thousands of discharges. Some of the *Gymnotids*, *Mormyrids* and others (LISSMANN, 1951; COATES, *et al.*, 1954; COUCEIRO *et al.*, 1955b) emit pulses continuously and at remarkably regular frequencies (Fig. 9). This is under control of their nervous system, as is shown by the fact that cooling only the head slows the discharge frequency of the organ (COATES *et al.*, 1954).[9] The eel, electrically silent when quiescent, also emits regular, relatively low-voltage pulses when cruising or hunting (cf. KEYNES, 1956).

IV. 4. *Central control mechanisms*

The discharge of the craniad and caudad portions of the electric organ of *Electrophorus* occurs so nearly simultaneously as to have led to the conclusion (COATES *et al.*, 1940; cf. also FESSARD, 1947) that the spread of the neural stimulus in the spinal cord developed at extraordinarily high conduction velocities of 1000 m.p.s. or even more. A more reasonable explanation has now been obtained along the lines suggested by FESSARD (1947). The midline nucleus of the central control located in the brain stem of the fish (COUCEIRO and FESSARD, 1953) send impulses down the spinal cord with normal conduction rates. These impulses in turn relay to the large efferent spinal cells, but the synaptic delay of the relay is high at the rostral regions, and shortens progressively for the more caudal relays (ALBE-FESSARD and MARTINS-FERREIRA, 1951, 1953; ALBE-FESSARD, 1954). This compensatory mechanism helps to account, at least in part, for the eventual synchronization of the discharge. A second mechanism is the effect of

[9] With similar experiments GARTEN (1910) had shown that the train of discharges reflexly elicited from *Malapterurus* was controlled from the brain.

current flows from discharging cells, which can enhance somewhat the excitability of other, as yet undischarged, cells.[10] A delay mechanism is probably also operative in *Torpedo* (ALBE-FESSARD, 1951b, 1952;

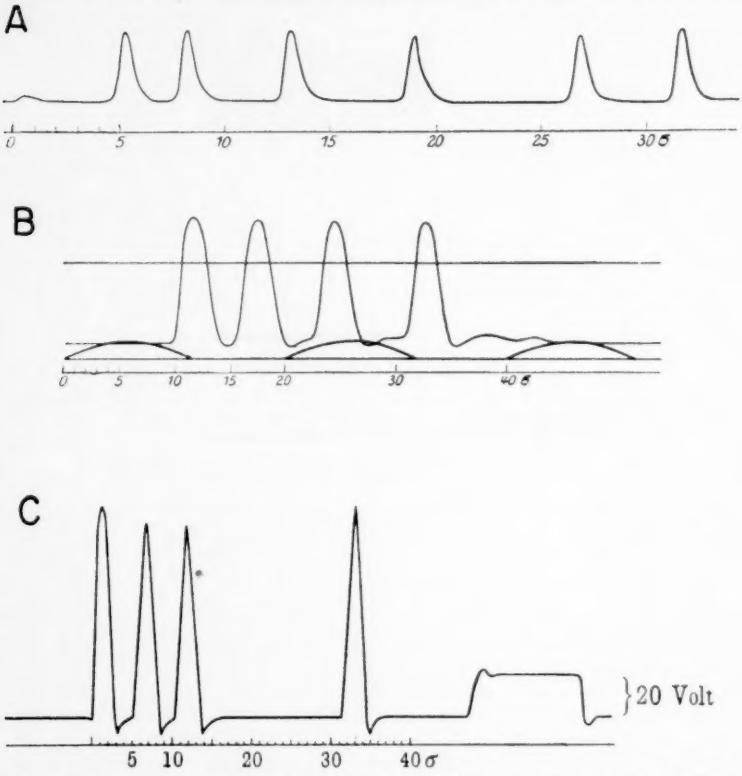

Fig. 8. Reflexly evoked discharges. *A*, *Electrophorus; B, Torpedo; C, Malapterurus.* In the first, a train is usually prefaced by a small discharge, probably representing chiefly a first, incomplete response of the main organ to the neural stimulus. The electroplaques excited by the first volley then probably respond maximally to subsequent stimuli. Although these records were made by CREMER with a galvanometer, using a heavy string, they are remarkably accurate, judging from the responses of *Electrophorus* and *Torpedo* obtained later with the cathode ray oscillograph, except that the undershoot in *C* was probably caused by the under-damped string as seen in the 20-volt calibration. (*A*, *B* from ROSENBERG, 1928; *C*, from GARTEN, 1910.)

cf. below), but *Malapterurus*, with its single axon dividing on its course from head to tail, presents an interesting puzzle, for the responses of the cells in this fish also appear to be well synchronized (Fig. 8).

The knife-fishes present an interesting range of variation in central controls.[11] Most of the species thus far examined emit pulses very

[10] This mechanism will be discussed below.

[11] Unpublished experiment in this laboratory with A. MAURO and E. AMATNIEK.

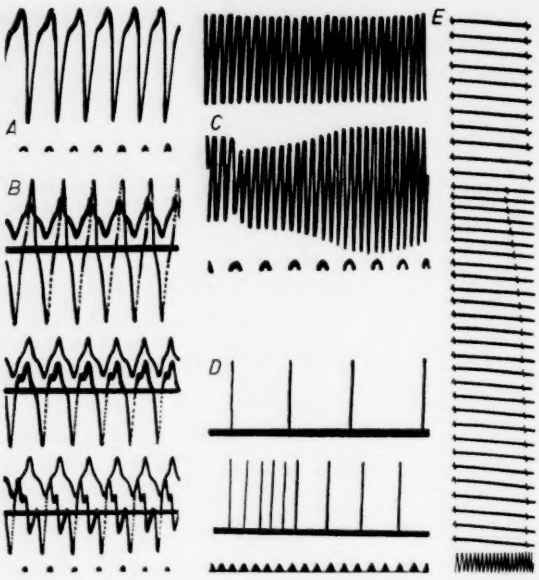

Fig. 9. Varieties of discharge in *Gymnotidae*. All experiments at room temperature *ca* 22°C. Negativity of the active electrode recorded as a downward deflection. *A. Adontosternarchus sachsii*, discharging at a rate of almost 900/sec, emits relatively simple pulses, predominantly negative at the tail. Time 1 msec. *B. Sternarchus albifrons*, discharge rate about 840 impulses/sec. The complex waveform is shown by the change in the different components at 3 recording sites. *Upper set*: The probe electrode and a reference standard (low gain) were both close to each other and at the tip of the tail. The third beam records the zero potential of the probe electrode. The probe was then moved several millimetres forward (*middle set*). The positive component decreased much more than did the negative. Another displacement of the probe led to the phase changes seen in the lowest set of records. Time 1 msec. *C. Eigenmannia virescens*. Frequency about 280/sec. Simple pulse, tail negative during discharge. A very strong electric shock caused the fish to struggle violently. This is reflected in the amplitude changes of the second record. However, the frequency of the discharges remained the same. Time 10 msec. *D.* Unidentified specimen (probably *Hypopomus artedi*) discharging tail-positive spikes at 2/sec. A tap on the aquarium wall caused the burst seen in the lower record, the frequency rising to about 10/sec, but slowing after a few discharges. Time 100 msec. *E. Gymnotus carapo*. Continuous record from above down, at about 25 sweeps/sec. The fish discharged diphasic spikes at about 50/sec. One of the pulses triggered the sweep and is seen at the beginning of each trace. A second appeared slightly before the end of the sweep, which lasted about 20 msec. The third again triggered the sweep, etc. The regularity of the discharge is fairly high as seen from the time of appearance of the second pulse on the sweep and from the spacing between two successive traces on the moving film. At the 12th sweep a gentle tap on the aquarium wall caused the discharge rate to increase, shown by the earlier onset of the second pulse and by the closer spacing of the traces. The return toward the initial rate was at first rapid, then slow. At the last trace shown, the rate was still about 5 per cent higher than before the stimulus. Time in milliseconds. (Data from unpublished work with Mr. E. AMATNIEK and Dr. A. MAURO.)

regularly and at frequencies ranging from about 1000/sec. (*Adontosternarchus sachsi*) to about 250/sec. (*E. Virescens*). Although this rate is centrally controlled (COATES, *et al.*, 1954), nevertheless it is independent of even violent movements caused by stimulation of the fish (Fig. 9). However, several species emit at low rates (ca. 2/sec.), and the frequencies of these discharges are temporarily increased when the fish is stimulated (Fig. 9). The rate of discharge, its form, and the degree of nervous control exerted, appear to be species specific.

IV. 5. *Role of electric discharges*

The purposive service of the discharges need be mentioned only briefly. The usefulness of the strong discharges for protection or aggression was noted earlier. Their value to those fish which develop only weak discharges, which are evoked only by stimulation (e.g. *Raia*), is less clear. It has been suggested that continuous emission of regular pulses by some of the electric fishes subserves locator functions, and the experiments do indicate that the fishes orient themselves well with respect to obstacles (cf. KEYNES, 1956). However, the distortion of electric fields produced by foreign-body inhomogeneities in a fluid medium must be rather small. If the fishes are capable of making use of that distortion it must be inferred that they possess extraordinarily sensitive receptors (COATES *et al.*, 1954). Study of these fish and their behaviour has not yet got to the point of providing information on such receptors or, if they exist, on their properties. The means of central control of this presumed locator mechanism are also unknown. In this connection an interesting, although negative, finding is the failure of *E. virescens* to alter its rhythm of discharge when external electrical pulses are delivered to the water. Shocks strong enough to cause violent movement of the fish do not disturb this rhythm (Fig. 9).

The problem of locator function is complicated by recent findings.[11] Some of the *Gymnotids* (e.g. *Sternarchus albifrons* and *G. carapo*) have several organs (cf. ELLIS, 1913, Pl. XIX, Fig. 20), in some species apparently differently oriented (Fig. 9). When recording with an exploring electrode at the tip of the tail of *S. albifrons*, the resultant response is a symmetrical diphasic potential, at first negative. Analysis of the potential field around the fish discloses that an anterior organ is oppositely oriented to the caudal. Nevertheless, when *S. albifrons* thrusts its head under a shelter in an aquarium, the tail portion extending out, the latter reacts to a metallic conductor brought into its vicinity, about 1 cm away from the skin. It does not react in the same way to a nonconductor. *Eigenmannia virescens*, which emits a monophasic pulse (Fig. 9) indicative of a single electric organ, is remarkably sensitive to a metallic conductor. When this is brought

near its body, a quiescent fish darts away, and continues these violent escape manoeuvres as long as a conductor, but not an insulated metal rod, is in its vicinity.

IV. 6. *Immunity of electric fishes from self-excitation*

This problem has not been attacked in modern times and our knowledge on this matter is not greater than that presented by Du Bois-Reymond in 1881. The latter pointed out that the electric organs are rather well isolated by fatty tissues, and that these also envelop the rest of the animal to insulate it somewhat from the discharge. The nerves to the electric organs are fairly heavily sheathed and are relatively difficult to stimulate or to record from in comparison with nerves from other animals. It is interesting in this connection to point out that the pair of single axons in *Malapterurus*, although only about 8–10 μ in diameter, are covered by about 1 mm of sheaths. The potential field generated in water by *E. virescens* indicates that the resistance of this animal forward of the electric organs is rather high (cf. footnote 11). These various items contribute to an explanation, but probably do not supply the full story.

IV. 7. *The chemical events and energetics of the electric discharges*

Very little detailed study of the chemical events is available. Release of acetylcholine during stimulation of *Torpedo* electric organ (Feldberg and Fessard, 1942) and disappearance of this substance during continued activity in *Narcine* (Chagas *et al.*, 1953b), and *Electrophorus* (Chagas *et al.*, 1953a), are known. The general biochemical processes of energy utilization have been studied to some extent in *Electrophorus* (cf. Nachmansohn *et al.*, 1943) and at least in gross are similar to those of muscle, breaking down phosphocreatine and releasing lactic acid. The rate of energy liberation is extremely high, a single discharge of *Torpedo* involving a peak of 6 kW and an average of 1 or 2 kW (Cox *et al.*, 1946; Crane, 1950). Electric organs provided material for an early study of the thermodynamics of bioelectric activity (Bernstein and Tschermak, 1906). The results, disclosing only small heat changes, sometimes exothermic, sometimes the reverse (cf. Bernstein, 1912) probably reflect the complexity of the biochemical sequence of events.

V. Electrophysiological Mechanisms of the Discharge

The earlier electrophysiologists, despite the sluggishness of their recording instruments, described essentially correctly the general properties of the discharges provoked by stimulating the animal, or the excised organs. Gotch (cf. 1900) and Garten (cf. 1910) observed a phenomenon confirmed by later workers (cf. Fessard, 1947) whose significance has been fully appreciated only recently. The response of

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the organs of *Malapterurus*, *Torpedo* or *Raia* to direct electrical stimulation always occurs with an irreducible minimum latency of several milliseconds. In 1882, BERNSTEIN (cf. GOTCH, 1900) had found that frog muscle stimulated through its nerve terminals also exhibits an appreciable minimal latency, whereas the response to direct stimulation of the muscle fibres can occur with almost no measurable latency. This finding led BERNSTEIN to distinguish different excitatory mechanisms in the two modes of response. Other data, the inexcitability of the denervated or curarized electric organs of these fishes even to the strongest stimuli, supported, in GARTEN's (1910) opinion, the view that the structures were end-plates, excited only by the intermediacy of neural action. The earlier experiments were confirmed and amplified by AUGER and FESSARD (1941), FESSARD and PÉZARD (1940), MARTINS-FERREIRA and COUCEIRO (1951), CHAGAS and ALBE-FESSARD (1954), and ALTAMIRANO *et al.*, 1953, 1955a,b.

V. 1. *Series summation of potential*

Despite the marked decrease of work on all electric organs after the beginning of this century,[12] and the virtual cessation of work on *Malapterurus*, a brilliant suggestion was made by BERNSTEIN (1912) to account for the high voltages which the electric fishes are capable of generating. Applying his theory that the electrical response of excitable tissues was caused by a temporary depolarization of the active region of a cell normally polarized with its interior negative, BERNSTEIN postulated that the electroplaque was similarly polarized, but that only its innervated surface membrane becomes depolarized during activity (Fig. 10).

The correctness in principle of this suggestion was shown only recently with intracellular recording from electroplaques of *Electro-*

[12] It is interesting to speculate on this decrease of attention paid to the electric organs. The development of sensitive and rapidly responding electrophysiological tools no longer made the strong generators of the electric organs convenient material. Research therefore probably tended to concentrate on the electrogenic tissues of greater potential interest to man, nerve and muscle. The First World War also played an important part. Apparently a considerable amount of work in progress on electric organs in Cremer's laboratory in Germany (cf. GARTEN, 1910; ROSENBERG, 1928), was disrupted by the First World War, only a few fragments of that work being published many years later (e.g. EILENFELD, 1926; REMMLER, 1930). In Japan also, the work of FUJI (1914) on *Astrape* was not resumed. Another factor which probably discouraged investigation was the lack of an accepted explanation for that most remarkable fact concerning the electric fishes—their unique ability to develop high-voltage activity. As noted in connection with VOLTA's "artificial electric organ," it was recognized, and especially pointed out by DU BOIS-REYMOND, that the probable cause was series summation of the e.m.f.'s generated in each electroplaque. However, until BERNSTEIN's suggestion, no explanation was forthcoming for this special adaptation of the electric fishes. As late as 1949 (ALBE-FESSARD *et al.*, 1949) leading workers in the field did not accept BERNSTEIN's explanation, and other mechanisms were proposed (AUGER and FESSARD, 1939; FESSARD, 1946).

phorus (Fig. 11) (KEYNES and MARTINS-FERREIRA, 1953; ALTAMIRANO *et al.*, 1955a,b) and *Raia* (BROCK *et al.*, 1953b) (Fig. 12). The response of the eel electroplaque to direct stimulation conforms with those of axons and skeletal or cardiac muscle fibres first clearly[13] demonstrated by HODGKIN and his colleagues (HODGKIN and HUXLEY, 1939, 1945; NASTUK and HODGKIN, 1950; DRAPER and WEIDMANN, 1951), and since found in many other conductile excitable cells. However,

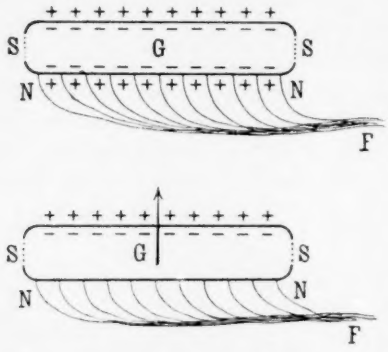

Fig. 10. BERNSTEIN's explanation of voltage summation in electric organs. The cell (*G*) is polarized at rest, inside negative. Impulses arriving in nerve trunk *F* act through the terminations *N* to depolarize only the innervated surface. This would set up a current flow in the direction of the arrow. S_1, *S* are the side walls. The regular arrangement of cells, one above the other, would act like an array of series batteries during the response, but at rest there would be no current flow. (From BERNSTEIN, 1912.)

only the membrane of the innervated surface of the electroplaque responds in this manner, temporarily not only losing its inwardly negative polarization, but the interior also reversing into positivity. The resting potential of about — 80 mV, and the overshoot to about + 70 mV, generate a pulse of about 150 mV in the eel electroplaque (KEYNES and MARTINS-FERREIRA, 1953; ALTAMIRANO, 1955). On the other hand, the noninnervated but electrically polarized faces of the cell show only a small potential during the discharge (Fig. 11). This is an IR drop of the current of activity across the resistance of the noninnervated and nonresponsive membrane (KEYNES and MARTINS-FERREIRA, 1953). Synchronous discharge of the approximately 6000 series-arrayed electroplaques of the eel therefore results in the high

[13] BERNSTEIN, in 1871, reported a reversal in nerve which DU BOIS-REYMOND (1881, p. 281) accepted. He therefore doubled his estimate of the size of the response of the eel electroplaque from a resting potential of 25 mV to activity of 50 mV. Apparently, on developing his depolarization theory in 1902, BERNSTEIN discarded his own earlier findings which were also neglected by other electrophysiologists. ENGELMANN made similar observations on heart, first published in 1873. BURDON-SANDERSON and GOTCH, in 1891, also reported this in muscle (cf. WEIDMANN, 1956, p. 19.)

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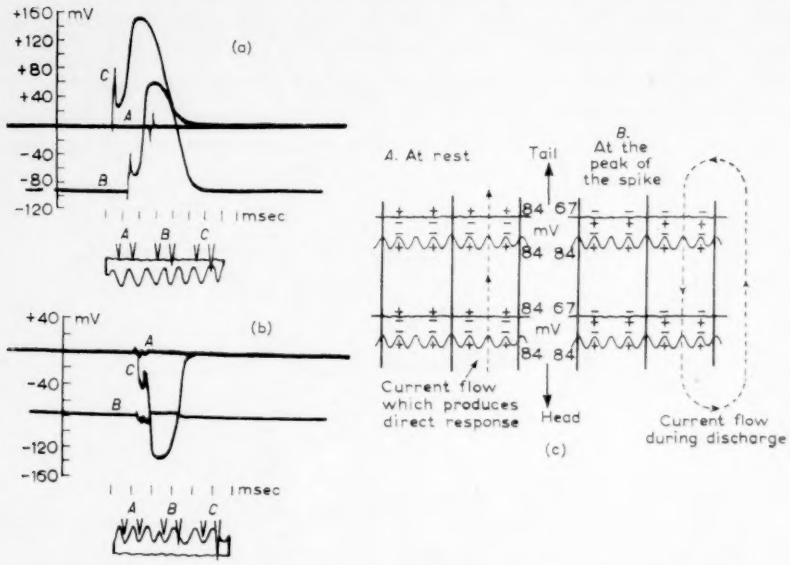


Fig. 11. Demonstration of the essential correctness of BERNSTEIN's explanation. *Upper left*: A pair of microelectrodes recording differentially and located at the innervated caudal surface of the eel electroplaque (*A* in diagram) are isopotential even when the cell is stimulated. One of the electrodes enters the cell to reveal a resting potential of almost — 90 mV. The response to a stimulus is a spike with overshoot (*B*). The resting potential disappears when the electrode is moved through the cell to the outside of the rostral surface, but the spike still appears in the same polarity (*C*). *Lower left*: The pair of electrodes are at first outside the noninnervated rostral surface (*A*). Entry of one electrode into the cell again discloses the resting potential (*B*), but a response elicits only a small IR drop across this membrane, showing that it does not generate a spike. On moving the same electrode through the cell to the outside of the innervated face, the resting potential disappears (*C*), but the spike remains, however, in reversed sign from that in *C* above, showing that only the outside of the innervated face becomes negative during the response. *Right*: diagram showing the alteration from the resting condition when no current flows in the electric organ, to the active, when the series arrangement of electrogenic surfaces produces a current flow and addition of voltages. (From KEYNES and MARTINS-FERREIRA, 1953.)

voltage of the whole organ, and of the current flow in the direction given by PACINI's rule—the innervated face becoming negative externally.

V. 2. *Parallel summation of currents*

Some of the electric fishes are also capable of delivering high currents. That estimated on short circuit of the eel organ is about 1 A, and of *T. occidentalis* is about 50 A (COX *et al.*, 1946). These currents are made available by the parallel arrangement of a number of series stacks (Fig. 2). *Malapterurus* is said to have some 3000 of these and *T. occidentalis* about 2000. The relatively smaller number of parallel

arrays in *Electrophorus* (up to about 70; cf. KEYNES, 1956) nevertheless are capable of delivering a high current because of the large surfaces of their electroplaques and the consequently low internal resistance of the individual generators. The innervated membrane has an apparent resistance of about 5 ohm-cm² (KEYNES and MARTINS-FERREIRA,

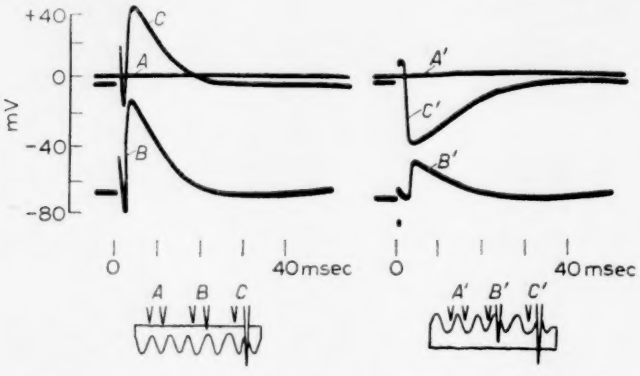


Fig. 12. Responses in the electroplaque of *Raia*. This cell is not excitable to an electrical stimulus, but is to a neural volley. The demonstration that only the innervated face of the cell responds to this stimulus is similar to that of Fig. 11. In this electroplaque, however, the activity does not have an overshoot (or not more than a few millivolts) and has a long duration (ca. 20 msec). (From BROCK *et al.*, 1953.)

1953; ALTAMIRANO, 1955), and the nonresponsive about 0·2 ohm-cm² (KEYNES and MARTINS-FERREIRA, 1953).[14]

V. 3. *Responses of eel electroplaque to electrical stimuli*

That the large response of the eel electroplaque is a spike (i.e. an action potential of the kind found in nerve or muscle fibres) is confirmed by its presence in a characteristic form in the denervated cell (Fig. 13; cf. MARTINS-FERREIRA and COUCEIRO, 1951). The direct electrical stimulus is then effective only when the current flows outward through the responsive membrane. With weak stimuli the first activity is a graded, local response, as in axons. This grows with stronger stimuli and a spike develops, propagating decrementlessly, but at a low rate (1–2 m.p.s.). With still stronger stimuli the latency decreases and nearly vanishes, but the stimuli tending to spread electrotonically excite the responsive membrane everywhere in the cell, and the dis-

[14] These values require correction for the increase in surface caused by its marked irregularity. PACINI estimated a 5- to 6-fold increase of the surface due to this factor (DU BOIS-REYMOND, p. 290). ALTAMIRANO (1955) suggests the increase may be up to 100-fold. The membrane capacity (ca. 15 μF/cm²; KEYNES and MARTINS-FERREIRA, 1953) would be decreased to the same amount as the membrane resistance is increased. However, the time constant, about 75 μsec, would remain unchanged.

charge of the latter then arises everywhere simultaneously or nearly so.[15]

The spike of the eel electroplaque is most probably caused by the

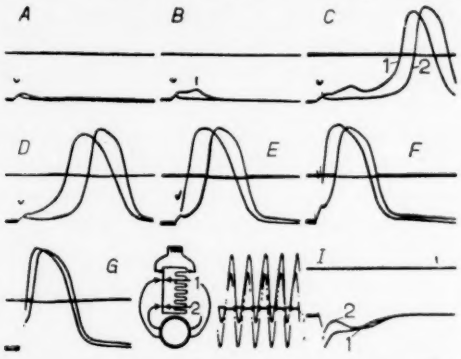


Fig. 13. Responses of the denervated eel electroplaque. The inset diagram shows the stimulating and recording conditions with 2 pairs of microelectrodes straddling the responsive caudal membrane of the cell. The upper of the 3 traces represents the zero potential base line. The resting potential deflects the 2 active traces downward. A weak stimulus (*A*), directed so that current flows outward through the excitable membrane (from right to left in the diagram) causes only electrotonic pick-up at both recording sites. A stronger stimulus (*B*) initiates a local response at the nearer recording site, 1. A stronger stimulus increases this, but still does not excite that region, the spike initiated at the stimulating electrodes propagating into recording site 1 and then into 2. Stronger stimuli (*D*, *E*) cause earlier onset of the response at site 1, since the exciting locus is moved closer, but that at site 2 arises only by propagation. Still stronger stimuli (*F*, *G*) spread farther, decreasing the latency at both recording sites and causing almost simultaneous discharge of the cell (*I*). Reversed, very strong stimuli do not activate the cell, the positive deflection being due to current flows in other, distant cells for which the stimulus was in the correct direction. Calibrations 100 mV and msec, shown separately for the two active traces. Upper is for trace 1. Spacings of the electrodes: between stimulating and 1, 0·83 mm; between 1 and 2, 2·56 mm. In subsequent figures of the same series only the values will be given, always in the same order. (From ALTAMIRANO *et al.*, 1955a.)

same type of membrane events which occur during the response of the squid axon (HODGKIN and HUXLEY, 1952), and which operate against a substrate of resting membrane potential,[16] and unequal distribution

[15] The responses of electroplaques of the main organ are similar (KEYNES and MARTINS-FERREIRA, 1953). The circuit properties of the eel-electroplaque are rather conducive to distortion of the recorded responses (ALTAMIRANO *et al.*, 1955a). This accounts for differences in amplitude and form of the spike observed under different conditions (KEYNES and MARTINS-FERREIRA, 1953; ALTAMIRANO *et al.*, 1955a; ALTAMIRANO, 1955).

[16] Failure to obtain a resting potential in *Torpedo* electroplaques led AUGER and FESSARD (1939) to propose a theory of electrogenic action different from that of BERNSTEIN. A resting potential up to about 60 mV has been observed by FESSARD and TAUC (1952), so that this cell, too, probably responds, as do eel and *Raia* electroplaques, by depolarization of the innervated membrane. However, some problems of the *Torpedo* response are discussed further on.

of ions between the cell and its exterior. Threshold membrane depolarization probably initiates a potential-controlled increase in sodium conductance. The influx of this ion, which is more concentrated in the body fluids than in the cell, depolarizes the membrane further and increases sodium flow still more.

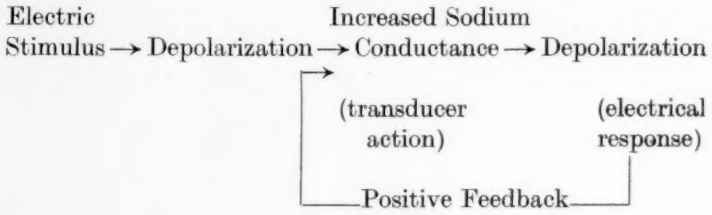
According to the HODGKIN and HUXLEY theory this regenerative process is halted by another, potential- and time-determined event, sodium inactivation. A third, similarly controlled process of heightened potassium conductance has kinetics which differ from those of either sodium conductance or sodium inactivation. This ion, flowing outward in conformity with its high internal concentration and the altered membrane potential, dissipates the membrane charge and potential.

The peak amplitude and the rate of rise of the spike of the squid axon depend upon the sodium concentration in the external medium (HODGKIN and KATZ, 1949). It has been similarly found (KEYNES and MARTINS-FERREIRA, 1953) that depletion of the Na^+ in the solution bathing the innervated surfaces of eel electroplaques depresses the spike. This finding, the existence of an overshoot, or reversed polarization of the membrane during the peak of the spike, and the loss of excitability in the depolarized electroplaque (Fig. 17) indicating the process of sodium inactivation, are evidence for the participation of sodium ions in the generation of the action potential of eel electroplaques.[16a] Although the kinetics of the processes may be different in the eel electroplaque (ALTAMIRANO, 1955; ALTAMIRANO *et al.*, 1955a), there seems to be no reason at present to assume ionic membrane mechanisms radically different from those postulated in the ionic theory developed for the response of squid giant axons. It has been suggested (ALTAMIRANO, 1955) that sodium inactivation and potassium conductance may be absent in the eel electroplaques. However, the increased membrane resistance of the electroplaque subjected to strong outward currents might indicate that a secondary process, perhaps one

[16a] Prof. KATZ has pointed out (personal communication) that OVERTON (1902) in his paper on the indispensability of sodium for the contraction of muscle had suggested (p. 381) examination of the responses of electric organs after replacement of sodium by sugar in the external medium. Presumably the success of BERNSTEIN's membrane theory, which was published in the same volume of *Pflüger's Archiv*, inhibited recognition of OVERTON's findings and his clearly stated conclusion that the response of muscle and probably also of nerve involves an exchange of Na^+ (or Li^+) for the intracellular K^+. It was first recalled to attention by LORENTE DE NÓ (1947), but its importance was not fully realized until HODGKIN and KATZ's (1949) fundamental work set the perspective. KATZ (1952) then pointed out the richness of OVERTON's paper. Another item of exceeding interest is OVERTON's discussion (pp. 383–384) of a difficulty for his suggestion. He pointed out that during a life span of 70 years the heart had contracted about 24×10^8 times and the respiratory musculature some 6×10^8 times. OVERTON noted that in spite of this activity there was no alteration of Na^+ and K^+ in the muscles of old people. To meet this difficulty he suggested that another mechanism (which is at present termed a metabolic "pump") probably existed to counteract the expected increase of internal Na^+.

of potassium inactivation, is disclosed by this finding. Heightened potassium conductance is said to be absent in the spike of frog axons (TASAKI and FREYGANG, 1955), but increased potassium outflow during repetitive activity is found in toad nerve (SHANES, 1951).

The behaviour of the electrically excitable membrane may be considered as a transducer action (GRUNDFEST, 1956b), depolarization acting to increase the sodium conductance according to the following schematization:



VI. SYNAPTIC EXCITABILITY

VI. 1. *Raia, Torpedines and Malapterurus*

The electroplaque of *Raia clavata*, on the other hand (Fig. 12), is not directly excitable, but is responsive to stimulation of its nerve (BROCK *et al.*, 1953b). The discharge always occurs after a latency of several milliseconds (ALBE-FESSARD, 1950a), is long (ca. 20 msec), and usually does not exceed the level of simple depolarization. If an overshoot occurs, it is only of a few millivolts. This response therefore comports with that originally suggested by BERNSTEIN. These properties, some of which had been earlier found with *Malapterurus* and *Torpedo* (cf. GARTEN, 1910) therefore re-enforce the view that the electrically inexcitable membrane of these electroplaques has the electrogenic properties of the muscle endplate as described by KATZ and his colleagues (FATT and KATZ, 1951; DEL CASTILLO and KATZ, 1954b). The response of *Raia* electroplaques is probably graded with different degrees of its excitatory innervation, and the amplitude can be smaller than full depolarization (cf. Fig. 12).

The discharge of *Raia* reflexly evoked through the bulbar command nucleus is a sustained potential similar to the tetanic response of muscle. It is probably due to repetitive stimulation of the organ by neural volleys emitted at intervals which are short in comparison with the duration of a single discharge of the cells (cf. SZABO, 1955). The sustained electrical activity is associated with graded responsiveness and absence of refractoriness. It therefore is to be expected in a p.s.p. Summation and maintenance of the p.s.p.'s is also to be seen in the responses of sympathetic ganglion cells which are stimulated so rapidly as to be incapable of supporting spikes (R. M. ECCLES, 1955).

VI. 2. *Electrophorus*

The innervated eel electroplaque[17] is responsive to a neural stimulus as well as to direct excitation, but the electrogenic activity is then different in some respects from that produced by the direct stimulus (Fig. 14). The neural volley evokes, simultaneously or nearly so all

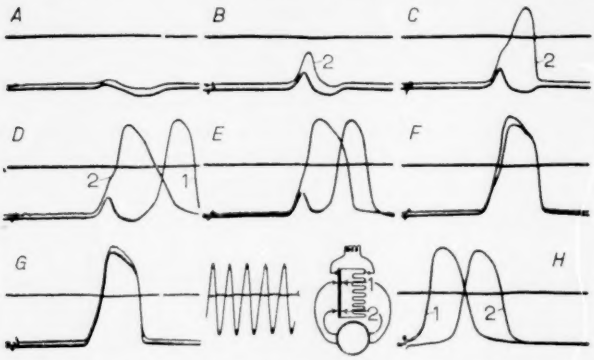


Fig. 14. The dual nature of neurally evoked responses of *Electrophorus* electroplaques. The arrangement for recording and for direct stimulation is similar to that of Fig. 13. Stimulating electrodes were also placed on one of the nerve trunks innervating the cell. This is not shown in the inset diagram. Increasing stimulation of the nerve evoked at first a graded depolarization (*A*, *B*) which grew more rapidly at the skin end of the electroplaque (site 2). The hyperpolarizing swing is due to current flows induced in the observed cell by the discharge of other cells, more excitable to the neural stimulus. The discharge of other cells in the series array is therefore inhibitory for the electrically excitable membrane, and this may account for the failure of propagation in *C* and slow conduction in *D*. Still stronger neural stimuli evoke a propagated spike arising near site 2 (*D*), but stronger nerve volleys excite the cell more widely (*E* to *G*), eventually causing its nearly simultaneous discharge everywhere. At all times, however, the spike is preceded by the initial step of depolarization which is absent in the directly elicited responses (*H*). Calibration 100 mV and msec, of both active traces. Distances 1·02 and 1·86 mm. (From ALTAMIRANO *et al.*, 1955a.)

over the responsive membrane, a small, graded potential which is usually largest at the lateral, skin end, of the cell. The amplitude of this potential grows when stronger stimuli are applied to one of the bundles of nerve fibres innervating the cell,[18] but is still largest at the skin end, and in that region evokes at first another localized response and then a spike. The latter then propagates along the electroplaque at the same velocity as the directly elicited spike. The initial graded response to the neural volley remains distinct from the spike recorded

[17] The responses of the single innervated cells have been studied thus far only in the Sachs organ (ALTAMIRANO *et al.*, 1953, 1955a,b; ALBE-FESSARD and CHAGAS, 1955a,b).

[18] Three of the segmental nerve trunks have been found to innervate a single electroplaque of the Sachs organ (ALTAMIRANO *et al.*, 1953). In *Torpedo*, six nerve bundles innervate each cell (Fig. 1).

in the more medial regions of the electroplaque. Still stronger stimuli to the nerve increase the early response more, and thus the spike may be initiated simultaneously everywhere in the cell. Nevertheless, a prefatory step in the spike still denotes the presence of the early,

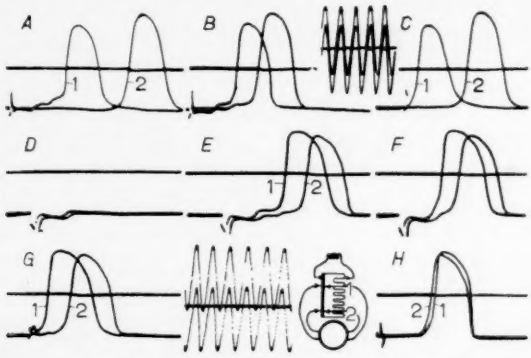


Fig. 15. Excitation of the innervated eel electroplaque with current flowing inward through its excitable membrane. As shown in Fig. 13, this does not elicit a response in the denervated cell and activation is therefore indirect, by exciting first the nerve terminals. A threshold stimulus (*A*) evoked an early depolarization near the site of the applied stimulus (site 1), the spike then propagating to recording site 2. (*B*) A stronger stimulus also caused a prefatory depolarization at site 2, the spike in the region arising by that excitation. (*C*) The cell directly stimulated by an outward current does not show these prefatory potentials. Note also the brief latency to a weak direct stimulus and the long latency to the indirect. Distances 0·83 and 4·5 mm. Indirect stimulation of another electroplaque is shown in (*D*) to (*F*). A weak stimulus (*D*) evoked a response in another cell which caused hyperpolarization of the observed electroplaque. A stronger stimulus caused depolarization prefacing the spike (*E*), and when the stimulus increased the prefatory potentials increased, initiating the spike earlier on the depolarization, the spike at site 2 also developing earlier than by propagation (cf. *G*). The directly elicited spike even to a very weak stimulus (*G*) arises with briefer latency than does the indirectly evoked (*F*), but the prefatory depolarizations of neural origin are absent. They are present in the discharge evoked by stimulation of a nerve trunk (*H*) although, as noted in Fig. 14, the spike in this case arises near site 2, and the cell discharges almost simultaneously. Calibrations 100 mV and msec. Distances 0·59 and 2·13 mm. (From ALTAMIRANO *et al.*, 1955a.)

neurally evoked, graded potential, which is not to be seen on direct stimulation of the cell.

Reversed "direct" stimuli which do not excite the denervated cell (Fig. 13) also evoke responses in the innervated electroplaque (Fig. 15). The spike so produced has a small and graded component prefacing it, as in the neurally evoked response. Even with very strong stimuli the response has a latency (ALBE-FESSARD *et al.*, 1951b). This form of stimulation has been termed "indirect" and is caused by stimulation of the nerve terminals at the surface of the electroplaque.

Various tests have demonstrated (ALTAMIRANO *et al.*, 1955a,b) that the

neurally or indirectly evoked responses always have the early component which is absent on direct stimulation, and that this component is a postsynaptic potential (p.s.p.) which develops in specialized, postsynaptic membrane of the electroplaque. It has also been shown that this membrane component differs in its properties from that of the electrically excitable portion of the same membrane (ALTAMIRANO *et al.*, 1955a,b).

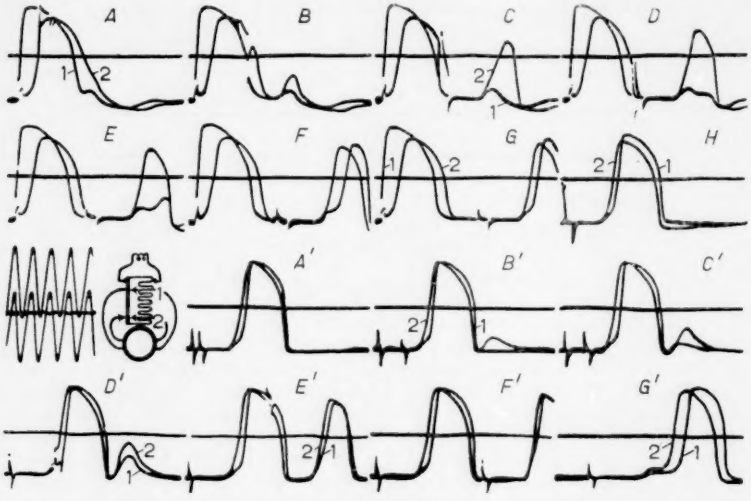


Fig. 16. Differentiation between the properties of the membrane developing the p.s.p. and the spike of eel electroplaques. Two kinds of experiments on the same cell are shown. *Above*: the cell was first excited with a direct stimulus, and this was followed at various intervals by a neural volley. The latter evoked a p.s.p. in the cell during the falling phase of the conditioning spike (*A*) and later (*B*) while the electroplaque was still absolutely refractory to a second, direct stimulus (cf. Fig. 27). When the neural volley arrived later, the cell was relatively refractory, and graded responses developed (*C* to *E*) which were largest at the skin end (site 2), where the synaptic excitation is strongest (cf. Fig. 14). Later, the cell became capable of propagating spikes (*F*, *G*), but was still relatively refractory, since these responses were small in amplitude and developed at site 1 with more latency than in the control response to the testing neural volley (*H*). *Below*: both the conditioning and testing response were evoked by stimulating the nerve. The testing stimulus was weaker than the conditioning as seen from the responses to this stimulus in isolation arising late on the p.s.p. (*G′*). The nerve being absolutely refractory to the second stimulus (*A′*), no p.s.p. was elicited by the latter. Early in relative refractoriness of the nerve (*B′*) the testing stimulus evoked a p.s.p. at the skin end where synaptic excitability is greatest. At longer intervals between the stimuli (*C′*, *D′*), a smaller p.s.p. developed and grew at site 1, that at site 2 always being larger. The spike-generating membrane was still absolutely refractory at this time (cf. record *B*, above). As the cell recovered into relative refractoriness (*E′*, *F′*) spikes were elicited, but smaller than the conditioning responses. However, they arose earlier on the p.s.p. evoked by the testing volley than when the latter was delivered to the unconditioned cell (*G′*), the effect being due to homosynaptic facilitation. The latter is not associated with persistent changes in membrane potential. Calibrations 100 mV and msec. Distances 0·59 and 2·13 mm. (From ALTAMIRANO *et al.*, 1955a.)

VI. 3. *Inexcitability of the postsynaptic membrane to electrical stimuli*

The fact that the p.s.p. is not obtained by direct stimulation of the cell suggests that this response is not generated in membrane capable of responding to an electrical stimulus. It has been found (ALTAMIRANO *et al.*, 1955a,b) that the membrane component generating the p.s.p. is indeed rather independent of the electrical conditions of the cell, whereas the membrane producing the spike is rather strictly subjected to these. The p.s.p. can develop even while the cell is not responsive to electrical stimuli either during the absolutely refractory period (Fig. 16) or when the spike generating membrane has been inactivated by

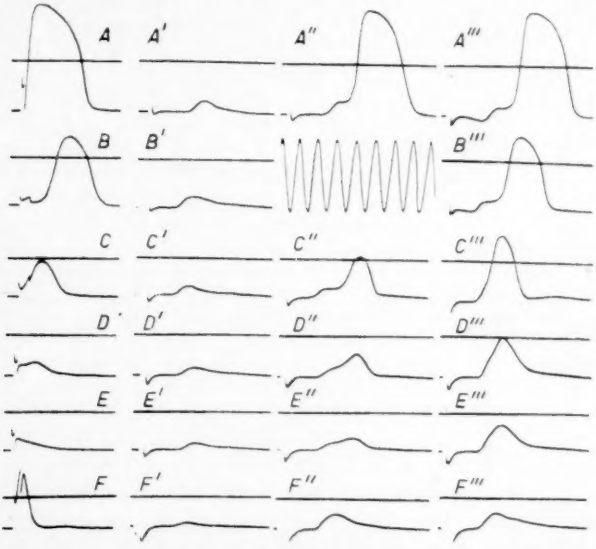


Fig. 17. Differential effects of depolarization on the spikes and p.s.p. of the eel electroplaque. Columns *A–F*, direct stimulation; columns *A′–F′*, etc., weak, moderately strong, and very strong stimuli to the nerve. *A–A‴*, the response of the normal cell. The strong direct stimulus evoked a spike with very brief latency (*A*). The weak neural volley caused a p.s.p. (*A′*) the stronger also a spike (*A″*, *A‴*). The cell was then treated with weak eserine (25 μg/ml solution) for 78 min, and weak acetylcholine (1 μg/ml for the last 58 min of that period. These drugs had no effect on the potentials. Five μg/ml acetylcholine were then added. Depolarization developed, the spikes 36 min later becoming smaller, but the p.s.p. was unaffected (*B–B‴*). The diminishing electrically evoked response 9 min later (*C–C‴*) became graded, as seen by its larger size in response to the strong neural volley. These effects progressed during the next 19 min (*D–D‴*) and 11 min thereafter (*E–E‴*). The p.s.p. to the threshold neural volley decreased (*E′*), but was still evident (*F′*) when the electrically excitable membrane no longer responded to a much stronger direct stimulus (*F*). The p.s.p. to a maximal neural stimulation (*F‴*) was still about as large as initially (*A‴*). This p.s.p. was still capable of evoking a small graded response of the electrically excitable membrane, as seen by the small delayed additional potential on the falling phase. Calibrations 100 mV and msec. (From ALTAMIRANO *et al.*, 1955b.)

depolarization (Fig. 17). Nor is the p.s.p. prevented by strong hyperpolarization of the cell which blocks appearance of a spike (Figs. 18, 19).

The sum of this evidence strongly indicates that the p.s.p. of the eel electroplaque develops in a membrane component which is not electrically excitable. This structure therefore probably resembles the

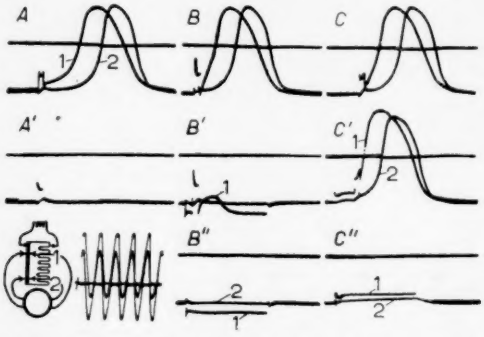


Fig. 18. Effects of hyperpolarization and depolarization on the electrically excitable membrane of an eel electroplaque. The excitatory effects of a weak stimulus (A) are eliminated by weak hyperpolarization of the cell(A'). A stronger stimulus (B) evokes only a local response at site 1 when the latter is subjected to about 15 mV of hyperpolarization (B') which is seen in isolation in B''. On the other hand, the excitatory effects of a weak direct stimulus (C) are enhanced (C') by depolarization of about 10 mV (C''). This behaviour is characteristic of electrically excitable membrane. Calibrations 100 mV and msec. Distances 0·83 and 2·56 mm. (From ALTAMIRANO *et al.*, 1955a.)

totality of the membrane in those electric organs which are only neurally excitable.[19] The muscle endplate is also endowed with this type of membrane (FATT and KATZ, 1951; DEL CASTILLO and KATZ, 1954b). An important new proof for the latter has been recently provided by the finding that even when the muscle fibre is depolarized and not responsive, the endplate membrane still reacts to application

[19] The "slow" muscle fibres of frog (KUFFLER and VAUGHAN WILLIAMS, 1953) do not give spike responses to electrical stimuli (BURKE and GINSBORG, 1955), their neurally evoked responses being entirely p.s.p.'s. The membrane of the apical dendrites of pyramidal cells in the cat cortex is electrically inexcitable (GRUNDFEST and PURPURA, 1956), but responds to synaptic activation (PURPURA and GRUNDFEST, 1956a,b). The pyramidal cells are excitable electrically at their soma. BREMER (1953, p. 4 footnote) remarks that an objection to the postsynaptic nature of synaptically evoked electrical activity is that, whereas antidromic excitation of motoneurons depresses the excitability of the cell, it does not much affect the "ability of a closely following orthodromic volley to produce a synaptic potential." This independence (cf. ALTAMIRANO *et al.*, 1955a) is exactly what may be expected if the postsynaptic membrane of neurons is not electrically excitable, and is to be seen in orthodromic responses of motoneurons immediately after antidromic excitation (BROCK *et al.*, 1953; COOMBS *et al.*, 1955c), in sympathetic ganglia (LAPORTE and LORENTE DE NÓ, 1950), and in other synaptic systems (cf. GRUNDFEST, 1956b). The p.s.p. of *Aplysia* ganglion cells also adds to a previously evoked spike, while the cell cannot respond with another spike (Dr. ARVANITAKI and CHALAZONITIS, 1956).

of acetylcholine (DEL CASTILLO and KATZ, 1955b). In the case of eel electroplaques (ALTAMIRANO *et al.*, 1955b) the neurally evoked p.s.p. develops even when the depolarized cell does not produce a spike (Fig. 17).

VI. 4. *Location of the post-junctional membrane*

The neurally evoked p.s.p. occurs everywhere that the spike does across the responsive membrane of the eel electroplaque. This graded

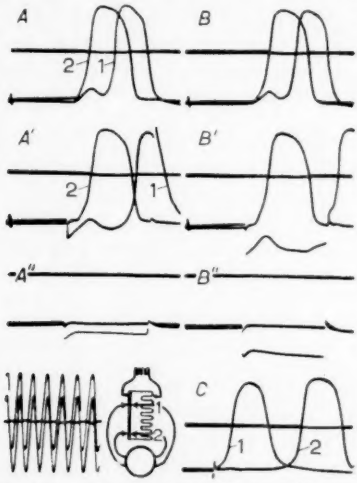


Fig. 19. Different actions of hyperpolarization on the membrane components generating the p.s.p. and spike of eel electroplaques. The response to a weak neural stimulus is shown in *A* and *B*. A hyperpolarizing pulse was then delivered through the electrodes of the inset, which caused a greater electrotonic effect at site 1 than farther away (site 2), as seen in records *A''* and *B''*. About 15 mV hyperpolarization slowed conduction into the hyperpolarized region (*A'*), and a change of about 35 mV increased this effect (*B'*). A delayed local response developed just before the hyperpolarization ended. In both cases, the p.s.p. elicited at site 1 remained essentially unaffected. The directly excited responses, devoid of p.s.p. are shown in *C*. Calibrations 100 mV and msec. Distances 0·83 and 4·5 mm. (From ALTAMIRANO *et al.*, 1955a.)

depolarizing response is recorded in opposite sign on the outside of the membrane (i.e. as increased negativity) just as is the spike. The two potentials must therefore be generated in the same membrane, and the latter must be a composite in which some "patches" give rise to the spike while others give rise to the p.s.p. In skeletal, "twitch" muscle fibres, the synaptically excitable membrane is concentrated at the endplate region and, as noted previously, the endplate membrane is not electrically excitable. In other cells also, relatively distinct regions with differently responsive membrane are known. The terminal dendrites of crayfish and lobster stretch receptors depolarize in response to mechanical or chemical stimulation (EYZAGUIRRE and KUFFLER,

1955a,b; WIERSMA *et al.*, 1953), the spike arising in electrically excitable membrane by electrotonic spread of the depolarization to this region (EYZAGUIRRE and KUFFLER, 1955a,b). However, the terminals are not themselves capable of supporting a spike (cf. also KUFFLER and EYZAGUIRRE, 1955). The soma of some cardiac ganglion cells of the lobster depolarizes to its synaptic excitation from the pacemaker cells of the ganglion, but the spike then develops in the axon (HAGIWARA and BULLOCK, 1955). The inability of cortical dendrites to respond to electrical stimuli or to support antidromic invasion by the spike of their cells (GRUNDFEST and PURPURA, 1956) is another case. On the other hand, the membrane of *Torpedo*, *Malapterurus* and *Raia* electroplaques is probably entirely of the synaptically excitable type,[20] whereas the eel electroplaque resembles that of cat motoneurons, in which the cell membrane is capable of generating a spike and also a p.s.p. In the latter cells, however, the postsynaptic membrane has more complications than the eel electroplaque, yielding not only depolarizing, excitatory p.s.p., but also a hyperpolarizing type—the inhibitory p.s.p. (BROCK *et al.*, 1952; ECCLES *et al.*, 1954; COOMBS *et al.*, 1955b,d). The latter has also been observed in some invertebrate muscle fibres (cf. FATT and KATZ, 1953) and in the crayfish stretch receptor (KUFFLER and EYZAGUIRRE, 1955). Further evidence that the postsynaptic membrane, in all systems at present accessible to study, is not electrically excitable, has been marshalled elsewhere (GRUNDFEST, 1956a,b,c).

VI. 5. *Pharmacological differentiation of the spike generating and postsynaptic membrane components*

As in *Malapterurus* and *Torpedo*, the eel electric organ is inactivated by curarization, but only to neural stimuli (DU BOIS-REYMOND, 1881, p. 197; CHAGAS and ALBE-FESSARD, 1954). A more extensive study, but still with only a few drugs, carried out with intracellular recording (ALTAMIRANO, *et al.*, 1955b) has provided further distinction between the membrane component generating the spike and that causing the p.s.p. The latter response is abolished by certain drugs (Table 1, Fig. 20), and with its disappearance there is loss of responsiveness to neural stimuli. However, the electrically excitable membrane component for a long time thereafter remains responsive, although the nature of its activity is altered, as will be described below. The effects of these drugs are exerted with no alteration of the resting potential, and therefore are not determined by potential-dependent processes such, for example, as sodium inactivation (HODGKIN and HUXLEY, 1952).

[20] Some invertebrate muscle fibres are not excited by electrical stimuli, but are responsive to neural (ROEDER and WEIANT, 1950; HAGIWARA and WATANABE, 1954; FURSHPAN, 1955; cf. also ALTAMIRANO *et al.*, 1955a).

Another group of substances, at least some of which are known to depolarize the muscle endplate (cf. ALTAMIRANO *et al.*, 1955b) also depolarize the eel electroplaque (Table 1, Fig. 17). This action of the

TABLE 1

(a) Compounds which inactivate the postsynaptic membrane, do not depolarize, but convert the all-or-nothing response of the electrically excitable membrane to the gradedly responsive

Substance	*Minimum effective concentration μg/ml*
Eserine	25
d-Tubocurarine	50
DFP*	100
Procaine	200
Tertiary analog of prostigmine	1000
Flaxedil†	—

(b) Compounds which depolarize, inactivating the electrically excitable membrane, but not the postsynaptic

Acetylcholine‡	5
Carbamylcholine	10
Decamethonium	10
Dimethylaminoethyl acetate (DMEA)‡	50
Prostigmine	50
Succinylcholine§	—

* This substance causes a secondary depolarization with consequent inactivation of the electrically excitable membrane.

† On the basis of the data of CHAGAS and ALBE-FESSARD (1954) that the action of FLAXEDIL is similar to that of curare. These workers did not study membrane potentials or graded responsiveness.

‡ In the presence of eserine, 25 μg/ml.

§ On the basis of the data of CHAGAS and ALBE-FESSARD (1954), who found a similarity of action with acetylcholine (see note †).

drugs is probably exerted on the postsynaptic membrane components of the electroplaque, the massive depolarization of these distributing itself over the totality of the membrane. It then affects the electrically excitable component, presumably by sodium inactivation, and the spike, whether elicited by direct stimuli or by neural, is depressed and finally abolished. At this stage, which represents loss of the all-or-nothing conductile property, strong neural stimuli still elicit a spike-like but not decrementlessly propagating potential. The action of the drugs on the p.s.p. is conspicuously different. Although there is some diminution of the threshold p.s.p. at late stages of drug action (Fig. 17) which suggest depression of synaptic excitability (cf. THESLEFF, 1955b), a maximal neural volley elicits the maximal p.s.p. of the normal cell.

The depolarizing action of the drugs in Table 1(*b*) is prevented by prior treatment of the electroplaque with one of the nondepolarizing synaptic blocking agents (CHAGAS and ALBE-FESSARD, 1954; ALTAMIRANO *et al.*, 1955b). Therefore both groups act upon the postsynaptic membrane component, and the effects of the synaptic blocking drugs is not due to some action of these on the presynaptic terminals. However, although the protected cell is not depolarizable by the drugs which usually depolarize, it nevertheless responds in this way to increased K^+ in the medium (ALTAMIRANO *et al.*, 1955b). It is not known whether the action

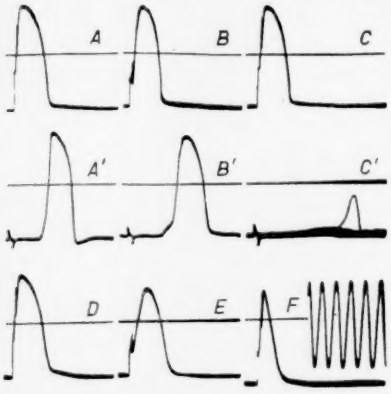


Fig. 20. Effect of nondepolarizing synaptic blocking agents on the responses of the eel electroplaque. Direct stimulation of the cell is represented in *A*–*F*, neural excitation in *A′*–*C′*. The initial responses to both stimuli are shown in *A* and *A′*. Three minutes after substituting a bathing solution containing 5 mg/ml d-tubocurarine, the directly elicited spike was unchanged (*B*), but synaptic excitation was less effective, the spike arising later on the smaller p.s.p. (*B′*). At 5 min (*C*, *C′*) the directly elicited response was still unaffected, but the p.s.p. had decreased so much (*C′*) that it was seen only with repetitive stimulation at 50/sec, and produced a single small "spike," after which it could no longer affect the electrically excitable membrane. The latter, however, remained fully responsive to a direct stimulus 41 min later (*D*), but eventually this responsiveness decreased (96 min later, *E*; and 110 min after this, *F*). The resting potential of the cell was unchanged. Calibrations 100 mV and msec. (From ALTAMIRANO *et al.*, 1955b.)

of the nondepolarizing drugs prevents depolarization of the postsynaptic membrane by K^+. Whether or not this be the case, the electrically excitable membrane still remains available for depolarization.

These pharmacological actions on the postsynaptic membrane of the electroplaque closely parallel those obtained with the same and related substances on the muscle endplate (cf. BURNS and PATON, 1951; DALLEMAGNE and PHILIPPOT, 1952; PATON, 1954). Indeed, the findings on the eel electroplaque preparation (ALTAMIRANO *et al.*, 1955b) constitute a demonstration, with the precision offered by intracellular

recording, of the correctness of the deductions from the studies with muscle and autonomic ganglia (PATON and PERRY, 1953). The bearing of the data on synaptic pharmacology is discussed below (Section IX).[20a]

VI. 6. *Presynaptically induced electrotonic currents in the electroplaque*

A curious finding is the absence of current flows which might be expected to develop in the richly synaptic surface of the eel electroplaque, electrotonically induced, after stimulation of the nerve, by the impulses of the presynaptic terminals. The e.p.p. of frog muscle also appears innocent of presynaptic electrotonic effects (FATT and KATZ, 1951; NASTUK, 1953a), while in motoneurons (BROCK *et al.*, 1952) or at the squid giant axon synapse (BULLOCK and HAGIWARA, 1955) only a brief, polyphasic electrotonic action is observed, in magnitude not exceeding a few millivolts.[21]

Correlation of these findings brings forward a number of possibilities. KUFFLER (1948, 1949) had indicated, and this was confirmed with intracellular recordings (DEL CASTILLO and KATZ, 1954a), that the electrotonic effects of the presynaptic volley are not sufficient to excite the muscle endplate. The absence, or small magnitude, of this effect in the electroplaque or other cells again supports this conclusion although, as has been described above, the postsynaptic membrane, being inexcitable by electrical stimuli is, in any case, not responsive to the electrotonically induced potential change.[22] However, the findings also indicate several additional deductions concerning the nature of presynaptic activity and of the synaptic preludes of the p.s.p.

[20a] ALTAMIRANO (1956b) has confirmed and extended our earlier joint work (ALTAMIRANO, *et al.*, 1955a,b, and unpublished experiments of that series). Results of particular interest are: supporting evidence that "depolarizing" drugs exert this action on the postsynaptic components of the electroplaque membrane; extension of the list of "nondepolarizing," synapse inactivating drugs; and evidence suggesting strongly that acetylcholine is not involved in the electrogenic responsiveness of electrically excitable membrane. Prolonged washing to the cells does not reverse the depolarization caused by acetylcholine or carbamylcholine, although the "nondepolarizing" compounds do so readily. As in other synaptic systems, the denervated electroplaque is more sensitive to acetylcholine (about 10 times more so) than is the innervated. Since the resting potential of unpoisoned cells is the same in both conditions (ALTAMIRANO, *et al.*, 1955a), the lower sensitivity of the innervated membrane to the drug cannot be ascribed to its "exhaustion" through bombardment with the transmitter. The difference is more probably due to a change in the chemical sensitivity of the denervated membrane (*cf.* Section IX, 4).

[21] Electrotonic pickups of similar magnitude are observed with intracellular recording from a quiescent giant axon when the nearby axons of the earthworm or crayfish nerve cord are active (KAO and GRUNDFEST, 1956, and to be published). The electrotonic effects of presynaptic impulses initiating the p.s.p.'s observed in the segmented axons of these forms are also small.

[22] Spikes can sometimes generate marked electrotonic potentials in other cells, as exemplified conspicuously by ephaptic excitation (ARVANITAKI, 1942), or by the excitability changes in one nerve fibre by activity in another (KATZ and SCHMITT, 1940).

VI. 7. *The significance of minimal synaptic delay*

When very strong indirect stimuli are applied to the innervated eel electroplaque (Fig. 21), excitation must occur in the electrically excitable portions of the presynaptic fibres nearest to the synapses, and conduction delay must then be at a minimum. Nevertheless, at least 1 and up to 2·5 msec elapse before a p.s.p. is seen (cf. Fig. 15).

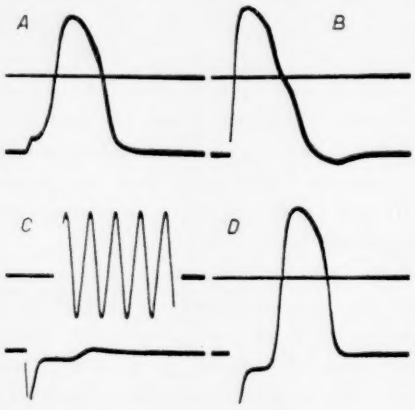


Fig. 21. Delay in synaptic excitation of the eel electroplaque. *Above*: Current flow outward through the responsive membrane, direct excitation. *Below*: Current flow inward, indirect excitation. The spike to direct excitation (*A*) arises with very brief latency when the stimulus is increased (*B*). On the falling phase in the latter case is a distortion which represents the p.s.p. evoked by the accompanying excitation of nerve terminals with the strong stimulus. A weak indirect stimulus (*C*) evokes only the p.s.p. which arises at the same time as does the distortion in *B*. A very strong indirect stimulus (*D*) elicits the spike only after a delay of 1·5 msec. Calibration 100 mV and msec. (From ALTAMIRANO *et al.*, 1955a.)

The delay in onset of p.s.p. may be, and in other contexts has frequently been, ascribed to slowed conduction in fine presynaptic terminals. Lateral spread of p.s.p. from the region of indirect excitation occurs in the electroplaque at about 5 m.p.s., indicating that the excited nerve branches close to the membrane conduct at this rate (ALTAMIRANO *et al.*, 1955a). It is therefore possible that the long latency preceding the p.s.p. represents an interval between arrival of the impulse in the presynaptic terminals, release of the transmitter, and its arrival and action at the postsynaptic surface. Another possibility is that the terminals are not themselves electrically excitable, their response in the form of

Geometrical considerations, the small terminal knobs impinging on a large surface of membrane, have been used to account for apparent absence of postsynaptic electrotonic effects. However, the density of innervation is high in eel electroplaques and probably much greater in those of *Raia*. Electrotonic effects also appear to be absent in the responses of *Raia* electroplaques (Fig. 12).

release of transmitter occurring on their electrotonic invasion by local circuit current from more central, electrically excitable regions. Slowed rise of the potential and its prolonged fall, associated with release of the transmitter, also in slowed tempo, would result in a synaptic delay, and would as well account for the absence of conspicuous electrotonic potential in the postsynaptic cell.

VI. 8. *Chemical vs. electrical excitation of the synapse*

The demonstration that the postsynaptic membrane of the eel electroplaque is not electrically excitable (ALTAMIRANO *et al.*, 1955a) forces the conclusion that synaptic transmission in this cell, as at the muscle endplate (KUFFLER, 1948, 1949; FATT and KATZ, 1951; DEL CASTILLO and KATZ, 1954a,b) cannot come about electrically through the action of eddy currents generated in the post-junctional cell by the impulses in prejunctional terminals.[23] As in the case of muscle and autonomic ganglia, it is therefore necessary to invoke a transmitter agent. The recent finding that the cortical dendrites of cat are not electrically excitable and cannot support antidromic invasion of an impulse (PURPURA and GRUNDFEST, 1956) is a new demonstration of obligatory chemical transmission. The nature of the transmitter of electric organs will be discussed below.

VI. 9. *Differential excitability of synaptic loci*

The larger magnitude of the p.s.p. at the skin end of the eel electroplaque than elsewhere in the membrane suggests that either the innervation of this region may be more dense or that the synaptic action is more powerful. Such differences are not observed in motoneurons probably because the cell is small and the potential developed by a given synaptic action tends to average itself by electrotonic spread over the entire membrane surface. However, other evidence does show differences of synaptic drives (e.g. LLOYD, 1955). These are usually ascribed to probable variations in density of synaptic innervation, but pharmacological evidence indicates that different cortical synapses may have different effectiveness or properties (PURPURA, 1956a,b; PURPURA and GRUNDFEST, 1956a,b).

VI. 10. *Interaction of repetitive synaptic volleys*

In the multiply innervated electroplaques it is possible to send a repetition of stimuli through the same nerve, thus exciting the same synaptic regions in succession (homosynaptic stimulation) or to interact the

[23] Illuminating discussions of the mode of synaptic transmission and of the pros and cons of electrical *vs.* chemical theories will be found in the following references: BREMER (1951), BREMER and BONNET (1949), BURGEN and MACINTOSH (1955), FATT (1954), FELDBERG (1950), FELDBERG and WHITTERIDGE (1955), FESSARD (1952), FESSARD and POSTERNAK (1950), ROSENBLUETH (1950).

effects of one neural pathway with those of another (heterosynaptic stimulation). The eel electroplaque is excited unequally by different paths (Fig. 22), and a single neural stimulus is conspicuously ineffective in the main organ.[24] However, although the neural volley discharges only a few cells it excites many more, and a second volley through the same nerve causes very marked homosynaptic facilitation (Fig. 23). The second stimulus is at first ineffective because the nerve is absolutely

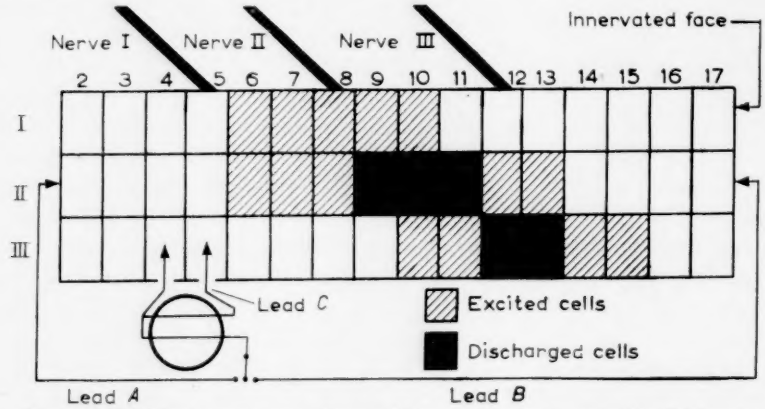


Fig. 22. Different excitatory effects of 3 nerve trunks innervating the same electroplaques of the Sachs organ. The same row of 16 cells is shown successively in I, II, and III. Excitation of nerve trunk I did not discharge any of these electroplaques, but facilitated the subsequent excitation of cells 6 to 10 by a second homosynaptic volley. Nerve II, innervated cells 6 to 13. A single volley discharged cells 9 to 11, but facilitated the response of cells 6 to 8 and cells 12 and 13 to a second volley from this nerve. Nerve trunk III excited cells 10 to 15, discharging cells 12 and 13 of these. External leads *A*, *B*, and *C* were the means used to identify these effects. (From ALTAMIRANO *et al.*, 1953.)

refractory, but after about 2·5 to 3 msec the response to the second stimulus grows larger than if it were not preceded by a previous conditioning volley, and rises at 10 to 15 msec to a flat peak which in the Sachs organ represents some 5 to 10 times more cells discharged by facilitation (ALTAMIRANO *et al.*, 1953) and in the main organ up to 50 times more cells (ALBE-FESSARD and CHAGAS, 1954). The effect may be seen even in a cell which is discharged by the first stimulus (Fig. 16). The facilitated response rises earlier on the p.s.p., and is capable of discharging the cell synchronously even during the relatively refractory period (cf. also ALBE-FESSARD and CHAGAS, 1955b). The time course of homosynaptic facilitation varies in different parts of the electric organ (ALBE-FESSARD and CHAGAS, 1954), but is characterized by an

[24] This was noted by SACHS (DU BOIS-REYMOND, 1881, p. 200) and was observed by ALBE-FESSARD *et al.* (1951a) and ALTAMIRANO *et al.* (1953). It has recently been studied in some detail by ALBE-FESSARD and CHAGAS (1954).

early, large component lasting about 50 to 80 msec, followed by a smaller residue (Fig. 23) which persists for more than 1 sec. There is no observable change in membrane potential during the facilitation (Fig. 16).

Heterosynaptic facilitation, on the other hand, is small and brief in the Sachs organ, as is the facilitatory interaction between a direct

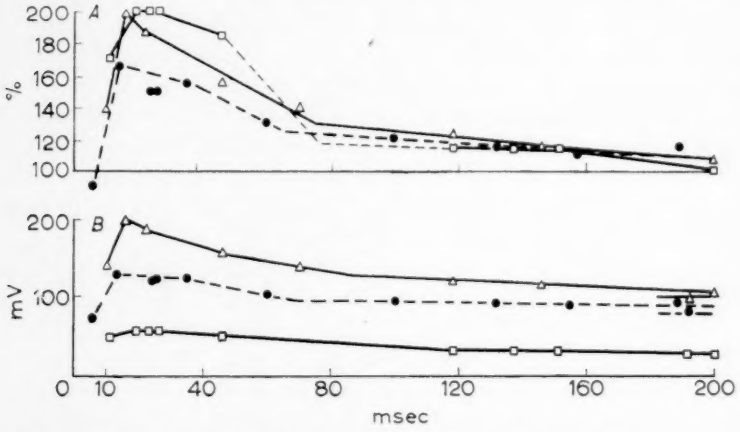


Fig. 23. The time course of homosynaptic facilitation in eel electroplaques and the effect on this of eserine. A strong conditioning neural volley excited the cells. A second, weak neural stimulus was evoked at various times thereafter. The height of the second response is shown (in millivolts) as the filled circles in *B*, that of this response in isolation (ca. 85 mV) is seen as the asymptote at the end of the curve. The percentile change of the response is shown in *A*. Some of the cells were refractory at about 5 msec interval between the stimuli, the test response being smaller than the control. A peak of facilitation, with the test response some 60 per cent higher than that of the control, developed between 10 and 40 msec after the conditioning stimulus, and fell to about 30 per cent at about 80 msec; but facilitation persisted more than 200 msec. Eserine (50 μg/ml) was then added. Twelve minutes later the drug had enhanced excitability to the testing volley, which produced a larger response to these in isolation (ca. 100 mV) seen as the asymptote to the upper curve of *B* (triangles). A larger amplitude of response was also produced on homosynaptic facilitation, but the time course of the facilitation was not significantly altered (*A*). The squares represent the facilitation 76 min after continued eserine action, when the drug had depressed neural excitability of the cells. Expressed in percentile terms, the relative facilitation was larger than previously, but the time course of the effect was the same. (From ALTAMIRANO *et al.*, 1955b.)

stimulus and a neural volley (ALTAMIRANO *et al.*, 1953, 1955a). These effects last about 2 msec. The main organ shows a somewhat larger amount of heterosynaptic facilitation between two neighbouring nerve trunks, but none between more distant nerves (ALBE-FESSARD and CHAGAS, 1954).[25]

[25] These authors also reported facilitation between direct and neural stimuli, but since have confirmed its absence (ALBE-FESSARD and CHAGAS, 1955b).

Long-lasting homosynaptic facilitation is also found in autonomic ganglia and, as in the electroplaque of the Sachs organ, heterosynaptic effects are small (LARRABEE and BRONK, 1947; JOB and LUNDBERG, 1953). Transmission at the partially curarized endplate is likewise facilitated by a second neural volley (HOFFMANN, 1903). Repetitive stimulation of the presynaptic nerve brings about a facilitation in muscle (FENG, 1941) and in motoneurons (LLOYD, 1949), termed by the latter "post-tetanic potentiation." The facilitatory effect is not seen when the conditioning repetitive stimulation is delivered to one presynaptic pathway and the excitability of the motoneurons tested with a second. Antidromic activation of the autonomic cell also does not leave behind facilitatory effects for a subsequent synaptic stimulus. This experimental condition is similar to interaction of direct and synaptic stimuli in the electroplaque, in which brief facilitation is seen because of exceptional conditions (ALTAMIRANO *et al.*, 1955a).

The differences observed between homosynaptic and heterosynaptic excitatory interactions have been ascribed (LARRABEE and BRONK, 1947; LLOYD, 1949; HUTTER, 1952; JOB and LUNDBERG, 1953) to increased effectiveness of the subsequent presynaptic stimulus, caused by the previous conditioning activity of the same nerve. The increase might come about by facilitated invasion of the impulse into previously inaccessible terminals, or by an increase in the amplitude of the presynaptic spike as a result of residual hyperpolarizing changes in membrane potential after the conditioning stimulation. Facilitation might then result from a larger release of transmitter at the individual terminations or by involving a larger number of terminals.[26]

An alternative suggestion considers the possibility of effects developed at the postsynaptic membrane (ALTAMIRANO *et al.*, 1955a; GRUNDFEST, 1956b). In the eel electroplaque, the phenomenon of homosynaptic p.t.p. may be said to occur after a "tetanus" of one neural stimulus (Figs. 16, 23). The synaptic transmitter released by the neural volley, once having exerted its primary excitant action of causing the p.s.p.,

[26] Most investigators of posttetanic potentiation (p.t.p.) apparently consider the presumed increase of the impulse as itself a sufficient cause for the facilitation, explicitly or implicitly accepting electrical transmission at the synapse. The peak of the spike of squid axons is not increased by hyperpolarization of the membrane, the overall increase of the impulse being caused only by its commencement from a lower starting level. If electrical transmission is excluded, p.t.p. as a presynaptic phenomenon requires the subsidiary assumption that more transmitter is released when the presynaptic terminal is hyperpolarized. Anodal polarization of the motor nerve facilitates the e.p.p. (DEL CASTILLO and KATZ, 1954a), and this provides a basis for presynaptic facilitatory action in terms of a chemical transmitter mechanism. HUTTER (1952) has presented what appears to be direct evidence for a presynaptic mechanism. While stimulation of the motor nerve produces a larger e.p.p. during the phase of p.t.p., the excitability of the endplate to acetylcholine is not increased. Therefore, the larger e.p.p. in the first case is presumably due to an increased amount of transmitter released on stimulation of the conditioned nerve.

might either persist at a low level of concentration or, combining with elements of the postsynaptic membrane, render the latter more excitable to subsequent release of transmitter by another neural volley. This facilitatory action should be spatially restricted, being largest at the sites of release of transmitter by the conditioning stimulus. The finding that heterosynaptic facilitation also exists in the main organ (ALBE-FESSARD and CHAGAS, 1954; noted also in the experiments of ALTAMIRANO *et al.*, 1953, 1955a), although absent in that of SACHS (ALTAMIRANO *et al.*, 1953) gives support to this interpretation, since the presumed presynaptic effects are then eliminated. Unfortunately, the kinetics of heterosynaptic facilitation have not as yet been studied. The presence or absence of heterosynaptic facilitation should be determined by the relative density and proximity of synaptic complexes. Presumably in the main organ these are sufficiently closely spaced so that transmitter released at one site reaches others innervated by a different nerve, and facilitates the excitatory action of the latter.[27]

Absence of a change in membrane potential during the period of homosynaptic facilitation (Fig. 16) may be an artifact of the recording conditions, since small, localized postsynaptic depolarizations would tend to average over the entire membrane surface. However, it is also possible that an altered chemical state of the postsynaptic component, evidenced by increased responsiveness (or lowered threshold) to the transmitter, need not be associated with a depolarization, which is itself a response to a stimulus. The temporary lowering of threshold to a neural stimulus which is produced in eel electroplaques by eserine or prostigmine (ALTAMIRANO *et al.*, 1955b) is not accompanied by depolarization.

The brief period of facilitatory interaction between a direct, electrical stimulus and a neural volley, on the other hand, is directly related to the membrane potential. The excitatory depolarization by the former sums with the depolarization of the p.s.p., but since both electrical effects are brief in the eel electroplaque, the facilitation is also brief (ALTAMIRANO *et al.*, 1955a). The equally brief period of heterosynaptic facilitation (ALTAMIRANO *et al.*, 1953) in the electroplaques of the Sachs organ indicates that this effect is also exerted by the interaction of the depolarizations developed by each synaptic stimulus, and is a result of their electrotonically summed actions on the electrically excitable membrane.

[27] The densely innervated electroplaques of *Torpedo*, each supplied by six distinct nerve trunks should prove an ideal system to test the merits of the different mechanisms proposed to explain homosynaptic facilitation. On the basis of the second it may be expected that large heterosynaptic facilitation should occur, while the contrary is the expectation if the facilitation is presynaptic in nature.

VI. 11. *Nature of the synaptic excitant*

Facilitation helps to provide some information concerning the nature of the transmitter. Eserine and prostigmine in low concentrations increase the synaptic excitability of eel electroplaques (ALTAMIRANO *et al.*, 1955b), although eserine eventually depresses and blocks the p.s.p. (cf. Fig. 23), while prostigmine does not. However, when these threshold changes were taken into account, the time course of homosynaptic facilitation was found to be unchanged (Fig. 23), and this, together with the absence of change in the amplitude or duration of the p.s.p. by low concentrations of anticholinesterases (ALTAMIRANO *et al.*, 1955b) and their different modes of action in high concentration (Table 1, Figs. 17, 20) suggest that the synaptic transmitter of the eel electric organ is not acetylcholine. Injection of acetylcholine does not discharge the eel electric organ (ALTAMIRANO *et al.*, 1955b) although it does that of *Torpedo* (FELDBERG and FESSARD, 1952).

However, the high concentration of cholinesterase at the innervated surface of the electroplaque (COUCEIRO *et al.*, 1955a), and the correlation between exhaustion of acetylcholine and fatigue of the neurally excited organ (CHAGAS *et al.*, 1953a,b) point to the cholinergic nature of this synapse.[28] A preliminary report (HARRIS and MIRANDA, 1955) states that homosynaptic facilitation is increased and prolonged by eserine. Furthermore, the depolarizing substances (Table 1) are of the type which exert this action at some cholinergic synapses. The possibility therefore remains open that acetylcholine is the transmitter agent of the eel electroplaque. The transmitter agent of *Torpedo* is probably acetylcholine, and the electrogenic action of this substance on *Torpedo* organ was one of the early demonstrations of that effect (FELDBERG and FESSARD, 1942).[29] *Narcine brasiliensis* utilizes acetylcholine during electric activity (CHAGAS *et al.*, 1953b) and it is therefore presumed that the transmitter is cholinergic. No data are available as to

[28] Fatiguability of synapses, which is so prominent in electric organs of the larger and stronger emitters, is usually ascribed to exhaustion of transmitter store. In *Narcine*, acetylcholine resynthesis appears to be very slow (CHAGAS *et al.*, 1953b) as compared with that in *Electrophorus* (CHAGAS *et al.*, 1953a). Fatiguability is conspicuously absent in the continuously emitting fishes (LISSMANN, 1951; COATES *et al.*, 1954; COUCEIRO *et al.*, 1955b; cf. Fig. 9), which presumably do so throughout their lives. The mechanism of these rhythmic, very regular discharges is discussed in Section VIII.

[29] As noted earlier, *Torpedo* electric organ is probably not electrically excitable. Therefore conclusions based on the electrogenic action of acetylcholine on this tissue (e.g. NACHMANSOHN, 1955) cannot be warrantably applied as support for its electrogenic action in electrically excitable membrane. Although often stressed by the same author, neither does the linear relation between quantity of cholinesterase and voltage output of *Electrophorus* organ pieces demonstrate his conclusion. The voltage varies with the linear density of electroplaques in series array (COX *et al.*, 1945). The larger the number of innervated surfaces per unit length, the greater the voltage. However, the cholinesterase is also concentrated at the innervated faces (COUCEIRO *et al.*, 1955a), and therefore the linear relation between voltage and quantity of cholinesterase indicates nothing more than that the latter is uniformly distributed among the electroplaques.

Raia or *Malapterurus*. The muscular origin of the electric organ in the case of all but the latter fish tempts the conclusion that acetylcholine is the synaptic transmitter in all of these.

Pharmacological data, at least in the present state of knowledge, do not provide decisive information about the nature of transmitters. For example, the curares are classical examples of agents blocking cholinergic synaptic systems. Yet, they also block many synapses of the mammalian central nervous system (OSTOW and GARCIA, 1949; PURPURA and GRUNDFEST, 1956a,b; GRUNDFEST and PURPURA, 1956a,b), but there is doubt as to which of these react to cholinergic transmitters (cf. FELDBERG, 1950; FELDBERG and WHITTERIDGE, 1955; BURGEN and MACINTOSH, 1955). Furthermore, synapses in different portions of the central nervous system, and even different peripheral systems known to be cholinergic, behave differently with respect to the curares and other blocking agents, and even different synapses on the same cells may be differently affected (PURPURA and GRUNDFEST, 1956a,b, and to be published).

VI. 12. *Nature of the postsynaptic membrane*

The pharmacological responsiveness of the eel electroplaque (CHAGAS and ALBE-FESSARD, 1954; ALTAMIRANO *et al.*, 1955b) is remarkably similar to that of the muscle endplate (cf. BURNS and PATON, 1951; DALLEMAGNE and PHILIPPOT, 1952), and suggests that the postsynaptic membranes of both have similar molecular structures. The fact that in cardiac pacemaker cells (DEL CASTILLO and KATZ, 1955c; HUTTER and TRAUTWEIN, 1956) the cholinoceptive[30] synapse is inhibitory and hyperpolarizing (or repolarizing, KUFFLER and EYZAGUIRRE, 1955) further indicates that the molecular components of both excitatory and inhibitory cholinoceptive postsynaptic membrane may have a common basic structure, modified somewhat to permit different electrogenic actions. The same kind of reasoning, based on the probable occurrence of adrenoceptive depolarizing or hyperpolarizing synapses in symmetrical inverse relation with cholinoceptive hyperpolarizing or depolarizing components, further suggests that the four classes of postsynaptic membrane—

adrenoceptive	*cholinoceptive*
hyperpolarizing	depolarizing
depolarizing	hyperpolarizing

have closely similar molecular structures, with modifications in each class which determine the specific excitant and the electrogenic action

[30] Since cholinergic and adrenergic refer to the action of the presynaptic nerves or of the transmitter analogs, the different synaptic membranes will be identified as *cholinoceptive* and *adrenoceptive* (DALE, 1954).

(GRUNDFEST, 1956b,d). An important new case in which the effects of dual autonomic innervation were studied with intracellular recording is that of the cat salivary-gland cells (LUNDBERG, 1955a). In some of these (Type II), stimulation of the parasympathetic nerves causes hyperpolarization, while the response to sympathetic stimulation is depolarizing. Type I cells respond with hyperpolarization to both kinds of stimuli, which probably indicates that the postsynaptic membrane (gradedly responsive here, too) contains only one component excited by both transmitters. The same hyperpolarizing response was obtained on injection of adrenaline, acetylcholine or pilocarpine. Cells of Type III, on the other hand, are depolarized by both kinds of neural stimuli. Facilitatory action by adrenergic transmitters on the endplate is well known (ORBELI effect, cf. HUTTER and LOWENSTEIN, 1955). Likewise, competitive actions between the excitant acetylcholine and adrenaline are found in sympathetic ganglia (MARRAZZI, 1939) and muscle (DALLEMAGNE and PHILIPPOT, 1952). The implications of these relations with respect to the above suggestion are discussed elsewhere (GRUNDFEST, 1956b,d; cf. also TOMAN, 1955).

When different types of p.s.p. are observed (as in motoneurons) it is generally taken for granted that these are generated by the action of different transmitters, released by nerves of different categories (cholinergic, adrenergic, etc.). This might not necessarily be the case. Since both hyperpolarizing and depolarizing responses to either acetylcholine or adrenaline are known, and these differences must reside in the nature of the postsynaptic membrane, it might also happen that acetylcholine liberated at one synapse of a cell would depolarize, while at another site it might act oppositely. As described earlier, the transmitter substances normally probably have only a limited zone of action. Therefore the existence of several types of p.s.p. can indicate the occurrence of different transmitters only if several subsidiary types of data (at present chiefly pharmacological) are also available.

The two types of action of the substances listed in Table 1 have been explained by postulating a "receptor protein" (ALTAMIRANO *et al.*, 1955c; WILSON and NACHMANSOHN, 1954) which is presumed to be involved in all electrogenic activity, including that of the electrically excitable membrane. In view of the fact that these substances apparently exert their blocking or depolarizing action only at the postsynaptic membrane, although at least some of them (e.g. d-tubocurarine) also enter into the electroplaque, as denoted by effects to be described below, the assumption is as yet not justified.[31]

[31] The spatial configuration of the "receptor protein" is assumed to be such that the latter is only partially affected by tertiary ammonium compounds, which hence are not depolarizing. The quaternary configurations are assumed to combine more effectively with the "receptor" to yield depolarizing changes. In the muscle endplate, neither d-tubocurarine nor pentamethonium depolarize, but reverse the effects of decamethonium

VI. 13. *Nature of electrogenic action at synapses*

All the available evidence at present indicates that electrogenic activity of living cells is caused by changes in membrane permeability which operate against a substrate of electrochemical gradients (HODGKIN, 1951; HODGKIN and HUXLEY, 1952) determined by the metabolic activity of the cell (cf. BROWN and DANIELLI, 1954). In the case of synaptic electrogenesis, however, the mechanism differs in detail from that causing the spike. This has been determined most clearly in the muscle endplate (FATT and KATZ, 1951; DEL CASTILLO and KATZ, 1954b, 1955b), and in the motoneuron (ECCLES *et al.*, 1954; COOMBS *et al.*, 1955a,b). The former system probably undergoes a general increase of permeability, leading to membrane depolarization as postulated by BERNSTEIN (1902), or to a level slightly below this (DEL CASTILLO and KATZ, 1954b).[32] The excitatory p.s.p. of the motoneuron appears to be due to a similar Bernstein depolarization, whereas the inhibitory potential is probably caused by a more selective permeability change either for Cl^- or K^+ or both (COOMBS *et al.*, 1955b), the entry of anions and/or the exit of the cations tending to drive the membrane potential toward greater internal negativity.

The data available on the eel electroplaque do not define the ionic changes associated with the p.s.p. However, the fact that this membrane can be completely depolarized by drugs (ALTAMIRANO *et al.*, 1955b) indicates that its electrogenic action also derives from a general increase of permeability. In *Torpedo*, which probably has only a synaptic response, the membrane resistance falls during the discharge (ALBE-FESSARD, 1950b).

The development of external negativity at the innervated face of all the electrically inexcitable types of electroplaques except those of *Malapterurus* indicates that in all of these the response is a depolarizing p.s.p. If the correlation of the physiological data and the innervation of *Malapterurus* organ is correct, the response of this exceptional form must be an increase of internal negativity, or what would be an inhibitory p.s.p., if these electroplaques had electrically excitable

(BURNS and PATON, 1951). In the eel electroplaque only the first has been tested with similar results. On the other hand, a tertiary compound is depolarizing (DMEA: Table I). As will be described below (Section IX) the "depolarizing" compounds have this effect only on postsynaptic membrane which responds with depolarizing electrogenesis. In oppositely electrogenic membrane the effect is of hyperpolarization. These compounds are therefore better designated as synapse activators. It is believed that certain strategic molecular configurations of drugs acting upon similarly strategic and specific structural sites of the membrane are responsible for drug actions. However, the relations between these molecular structures and pharmacological actions are as yet not clear.

[32] The increased conductances of all the major ions might occur with different kinetics. Were the rate higher in the specific case of Na^+, an overshoot might then develop to give the appearance of a response resembling a spike. This will be discussed below in connection with the responses of *Torpedo*.

membrane to inhibit. The presumably different origin of these electroplaques, from secretory gland cells of the skin, rather than muscle, has suggested (GARTEN, 1910; cf. also ROSENBERG, 1928; KEYNES, 1956) that their reversed electrogenic response is associated with secretory action. LUNDBERG's (1955a) finding that secretory activity of some salivary gland cells is associated with hyperpolarization supports this possibility. Selective ion movements of secretory cells are: Na^+ in frog skin (USSING, 1954) and Cl^- in gastric mucosa (HOGBEN, 1955).

The changes of ionic permittivity at the postsynaptic membrane induced by the specific stimuli of transmitters can be schematized as transducer action (GRUNDFEST, 1956b):

Specific chemical stimulus	→	Change in membrane permeability (transducer action)	→	Membrane depolarization or hyperpolarization (electrogenic action)

Since the transducer action is not electrically excitable, the regenerative property which leads to the all-or-nothing spike is absent, and graded responsiveness is a characteristic of the activity.

Presumably each molecular "valve" or "carrier" which becomes activated by the synaptic transmitter operates in an all-or-nothing fashion, and were one to probe that region with a "micro-molecular" electrode the membrane presumably would register at each "valve" region the electrode potential of the ion involved. However, electrotonic spread of the effects of the individual changes averages into a potential which is usually smaller than full depolarization or hyperpolarization to the maximum level of the electrochemical potential of the ion involved. In the case of the *Raia* electroplaque the level of zero membrane potential is attained in the response (BROCK *et al.*, 1953b).[33]

The p.s.p. of the eel electroplaque does not alter conspicuously for a change in membrane potential of about 40 mV either way (Figs. 17, 19), whereas the e.p.p. of muscle or the p.s.p.'s of motoneurons are more readily affected by the membrane potential (FATT and KATZ, 1951; ECCLES *et al.*, 1954; COOMBS *et al.*, 1955a,b). The reason for this difference has not yet been adequately examined. The response of *Torpedo* electric organ is decreased on depolarization of the membrane and increased on moderate hyperpolarization (ALBE-FESSARD, 1951a). Since these experiments were carried out with external recording from a large group of cells, the effects of variation in excitability of the nerves and a consequent variability in number of responding electroplaques could not be excluded, nor could the level of membrane potential be

[33] If the occasional observation of a few millivolts overshoot is not due to experimental errors, its occurrence might be accounted for by slightly greater rates of rise in the Na^+ conductance as discussed in VI, 14 (p. 47).

determined. However, the data are highly suggestive of the similar behaviour of endplates and neuronal synaptic membrane. The response decreases with increasing membrane depolarization and even reverses (Fig. 24; cf. DEL CASTILLO and KATZ, 1954b; ECCLES *et al.*, 1954; COOMBS *et al.*, 1955b; BURKE and GINSBORG, 1955). Also (Fig. 17, p. 69 of the paper) stimuli which do not elicit an electrogenic response nevertheless decrease the membrane resistance (cf. DEL CASTILLO and

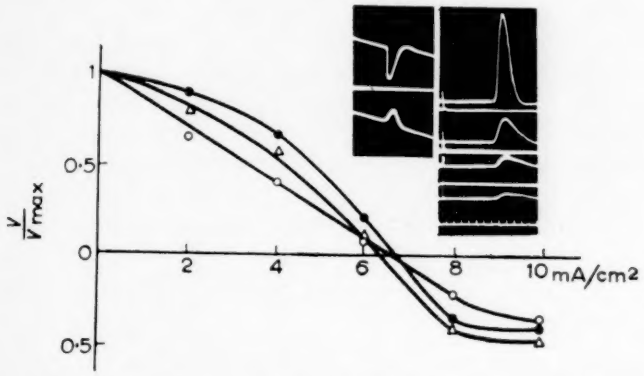


Fig. 24. Changes of the response of *Torpedo* electric organ subjected to various strengths of depolarizing current. *Right*: The portion of the organ produced about 12 V on discharge (upper record). The response decreased with progressively increasing depolarizing currents. *Left*: The upper record is the response without a depolarizing current (recording connections reversed from the previous set). An extremely strong current inverted the response. The graph shows three sets of experiments, the applied current on the abscissa and the ordinates representing the discharge voltage as a fraction of the initial response. In all cases the sign of the discharge was inverted with strong applied currents. This behaviour does not correspond with that of a spike, but can be ascribed to a p.s.p. (Composed from ALBE-FESSARD, 1951a).

KATZ, 1955b). These data which appear difficult to explain if the response were a spike therefore support the view that the response of the *Torpedo* electroplaque is a p.s.p.[34]

[34] ALBE-FESSARD (1952) has suggested that the synchronization of the response in various parts of the *Torpedo* organ is due to combined action of neural factors and of a facilitation of excitability produced in cells not yet responding by cells which are already discharging. Such facilitation is unlikely even from electrical considerations. In the electric organ of all but *Malapterurus* the action current runs inward with reference to the responsive membrane (Fig. 7). This direction is not effective for the electrically excitable membrane of eel electroplaques (Fig. 13). In-series elements therefore tend to be anodally polarized. Only electroplaques in parallel may be facilitated, but their contribution to the total recorded voltage should be less than that of the in-series cells. On the basis of the view proposed here, that the response is postsynaptic (FESSARD, 1952) and therefore not electrically excitable, facilitation would not occur by eddy currents, but would come about through heterosynaptic facilitations among the effects of the six nerve bundles innervating each electroplaque, and perhaps also of homosynaptic facilitations in repetitive responses of different nerves. This matter cannot be resolved with the available data.

VI. 14. *Magnitude of the p.s.p. in different electroplaques*

The small magnitude of the p.s.p. in eel electroplaques has been noted and discussed above. It was also pointed out that treatment of the cell with drugs can bring about full depolarization. Therefore, presumably, the neural volley releases relatively little transmitter and the transmitter is probably also evanescent. The response of *Raia* electroplaques, which are much more densely innervated than those of *Electrophorus*, is capable of full depolarization (BROCK *et al.*, 1953b). In the *Torpedines* and *Malapterurus* the e.m.f.'s of individual cells may be roughly estimated from the maximal discharges of the animals and from the number of series elements (Table 2; cf. also ROSENBERG, 1928; FESSARD, 1947). These values are very high and apparently contraindicate the synaptic nature of the response.

TABLE 2*

	Series elements	*Maximal discharge*	*EMF*
1. *Electrophorus electricus* . .	ca. 6000	886 V	ca. 150 mV
2. *Raia clavata* . . .	,, 200	4 V	,, 20 mV
3. *Torpedo Marmorata* . .	,, 400	45 V	,, 110 mV
4. *Narcine brasiliensis* . .	,, 300	37 V	,, 120 mV
5. *Malapterurus electricus* . .	,, 1600	350 V	,, 220 mV
6. *Astroscopus guttatus* . .	,, 200	50 V	,, 250 mV

* The sources for this table are given by KEYNES (1956), except that the latter lists about 650 V as the maximal discharge of *Electrophorus*. That cited by ROSENBERG (1928) is used here. ROSENBERG (p. 900) estimated about 140 mV as the e.m.f. of the eel electroplaques, and FESSARD (1947, p. 24) estimated that of *Torpedo* electroplaques as 100 mV. GOTCH and BURCH (1899) estimated the e.m.f. of *Malapterurus* electroplaques at about 48 mV, but this involved the assumption that 2 columns of cells dovetailed. Otherwise their data yield a value of about 100 mV. However, all the data of older vintage (e.g. DU BOIS-REYMOND, 1881; GOTCH, 1900) give estimates which are too low.

It will be noted that in the case of *Electrophorus* and *Raia* the e.m.f.'s calculated for the individual cells are similar to or lower than those directly determined, so that the estimates for the other electric fishes might actually be too low. Nothing is known about the electrophysiology of *Astroscopus*, which may have a spike response, nor are there relevant data available for *Narcine*, although its relation to the other *Torpedines* would suggest that the response should be a postsynaptic potential. The e.m.f. of 110 mV calculated for the electroplaques of *T. marmorata* might indicate an especially high resting potential and full depolarization to this level as in *Raia*. However, FESSARD and TAUC (1952) obtained resting potentials of 45 to 60 mV

which would seem to indicate that the response of the *Torpedo* electroplaque is greater than depolarization.[35] The likelihood therefore that these cells respond with an overshoot cannot be dismissed. However, this response might still be a p.s.p., as all other types of data indicate it is. That might come about, as noted on p. 44 (footnote 33) if the heightening of sodium conductance occurred before the increase of K^+ or Cl^- conductance. If the former process considerably preceded the latter events (as it does in the spike), a large overshoot might be obtained in a postsynaptic response. A crucial test of the nature of the response could be obtained from intracellular recordings of *Torpedine* electroplaques. If this is a p.s.p. it should be possible to grade the amplitude, particularly as each plaque is provided with six separate nerve supplies. Presumably this would not be the case in *Malapterurus*, in which a single axon is supposed to innervate all the cells of one side. However, in these, as in *Torpedo*, the response might reverse under suitable change of electrochemical conditions. In the absence of intracellular recordings and of further knowledge concerning even the nature of the response of *Malapterurus* we can resolve this matter no further.

VI. 15. *Form of the p.s.p.*

Nor can the nature of the responses, whether these are spikes or p.s.p.'s in the cases discussed above, be resolved by considering their durations (cf. FESSARD, 1947). The densely innervated electroplaques of the *Torpedines* probably respond maximally to stimulation of their nerve trunks. In *Torpedo* and in *Malapterurus* the response lasts about 2 to 3 msec (Fig. 8), and therefore has the appearance of a spike, in comparison with the long-lasting potential of *Raia* (ca. 20 msec, Fig. 12). However, the p.s.p. of *Electrophorus* is as brief as the spike (Fig. 14), in contrast with the long p.s.p.'s of neurons, muscle, or secretory cells of the cat. The duration of the p.s.p. of the squid giant axon is also short (BULLOCK, 1948; BULLOCK and HAGIWARA, 1955).

Transmitter action, until better known, provides too much flexibility in conditions to determine precisely the reason for the existence of long and short p.s.p.'s. FATT and KATZ (1951) and ECCLES and his colleagues (BROCK *et al.*, 1952; ECCLES *et al.*, 1954; COOMBS *et al.*, 1955a,b) have made the reasonable assumption that, if the transmitter

[35] A resting potential of 60 mV has also been obtained in our experiments (unpublished work with Dr. C. Y. KAO). Errors in measurement of resting potential are not to be minimized. Even in simple cases such as squid or lobster giant axons, the spread of observed resting potentials is rather high (e.g. GRUNDFEST *et al.*, 1954; TOBIAS, 1955), and similar findings are observed whenever the data are sufficiently extensive (e.g. BURGEN and TERROUX, 1953; FRANK and FUORTES, 1955). The extreme cases are those in which no resting potential is observed, as in marine eggs (e.g. ROTHSCHILD, 1938; LUNDBERG, 1955b; cf. GRUNDFEST *et al.*, 1955; TYLER *et al.*, 1956). Even though the latter workers found a resting potential in *Asterias* eggs, this declined rapidly from a maximum value of about 65 mV to about 30 mV.

is released suddenly and acts for a brief time, the p.s.p. becomes essentially a ballistic response. The amplitude would then be determined by the quantity and potency of the transmitter, but distorted somewhat by the electrical-circuit properties of the membrane, including averaging by electronic spread. The decay of the response should reflect the time constant of the passive circuit of the resting membrane resistance and capacity. This seems to be approximately the case for the e.p.p. and the p.s.p.'s of neurons. However, the time constant of the eel electroplaque is very brief (ca. 0·1 msec; KEYNES and MARTINS-FERREIRA, 1953) while that of the squid giant axon is about 1 msec (cf. COLE and CURTIS, 1950), yet the durations of the p.s.p.'s (about 2 to 3 msec) are similar in both.

Prolongation of transmitter action at the muscle endplate by anticholinesterases leads to prolongation of the e.p.p. (FATT and KATZ, 1951). This is not the case in eel electroplaques. In the former, also, deprivation of Na^+ results in a more prolonged response, but comparable data on the electroplaque are as yet lacking.[36]

VI. 16. *Transfer of excitation from the postsynaptic to the electrically excited membrane*

Although the principle is the same as that observed in the frog muscle fibre (FATT and KATZ, 1951; NASTUK, 1953a) somewhat more detail is available from a study of the process in the eel electroplaque, because it is possible to grade the p.s.p. by varying the strength of the neural volley (ALTAMIRANO *et al.*, 1955a). The p.s.p. generates a local response in the immediately adjacent electrically excitable membrane (Fig. 25). The two graded potentials combine, normally being so fused as to be indistinguishable. However, the local response is subject to the refractoriness of the cell, whereas the p.s.p. is not. The local response therefore arises later and becomes smaller as a neural volley arrives successively earlier during refractoriness of the electroplaque, and finally disappears during absolute refractoriness. The "pure" p.s.p. then remains.

Graded stimulation of the nerve supply leads to graded, perhaps quantal, responses (cf. FATT and KATZ, 1952) of the postsynaptic membrane (Fig. 26). These accumulate as the stimulus is increased and eventually become large enough to elicit a local response and a spike. Gradation of the p.s.p. is also observed in motoneurons (BROCK *et al.*,

[36] Presumably, choline or other complex ions substituting for Na^+ can penetrate the cell membrane. Otherwise, were K^+ and Cl^- alone mobile, the e.p.p. should reverse to hyperpolarization, or become insignificant if the resting membrane is already near the equilibrium potential for the two ions. Permeability of such ions through the excitable membrane of some nerve fibres (LORENTE DE NÓ, 1949), but not of others, would also account for the responses observed in the former on sodium deprivation substituted by the organic molecules.

1952; ECCLES *et al.*, 1954; COOMBS, *et al.*, 1955b,c,d; ECCLES, 1955), and in secretory cells (LUNDBERG, 1955a). Quantal "miniature" responses (FATT and KATZ, 1952; DEL CASTILLO and KATZ, 1955b) have not been observed as yet in the eel electroplaque. However, the

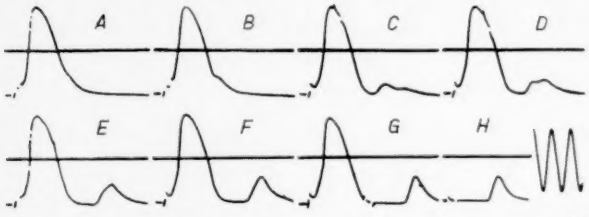


Fig. 25. Transfer of excitation in the eel electroplaque from the p.s.p. to the electrically excitable membrane *via* local response. The cell was not discharged by the strong neural volley, but a p.s.p. potential was evoked (*H*). However, the depolarization was composed of two elements, the p.s.p. itself and a graded response in adjacent electrically excitable membrane. The latter disappeared (*A*, *B*) when the neurally evoked response fell during the absolutely refractory period following a previous response, although the p.s.p. remained and is visible on the falling phase of the conditioning spike. When the neural volley arrived during relative refractoriness of the cell a late component of graded response could develop (*C*, *D*). During further recovery of the cell the graded response developed earlier on the p.s.p. (*E*, *F*), fusing with the latter (*G*). Calibrations 100 mV and msec. (From ALTAMIRANO *et al.*, 1955a.)

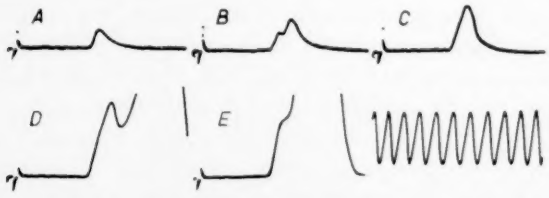


Fig. 26. Transfer of excitation from the p.s.p. to the electrically excitable membrane; gradation of the p.s.p. up to a critical level of depolarization for initiating a graded response and spike. A weak stimulus to the nerve produced a small component of p.s.p. (*A*) to which a stronger stimulus added a second quantity (*B*). The p.s.p. reached almost the critical firing level in *C*, exceeded it in *D*, and was just adequate in *E*. Calibration 10 mV and msec. (From ALTAMIRANO *et al.*, 1955a.)

distribution of synaptic sites in the latter, probably punctiform and dispersed in comparison with the massive synaptic region of the end-plate, would make observation of "miniature" p.s.p.'s difficult in the eel electroplaque. The *Torpedine* cells, on the other hand, should provide good experimental material for this problem.[37]

[37] The evidence of KATZ and his colleagues (FATT and KATZ, 1952; DEL CASTILLO and KATZ, 1956) indicates that quantal miniature e.p.p.'s are discharged by small releases of transmitter from the presynaptic terminals. However, attention might also focus on the possibility that thermal agitation of the postsynaptic membrane could also cause occasional molecular configurations of the latter which would permit ionic

The dispersed distribution of p.s.p.'s in the eel electroplaque also limits their ability to "short circuit" the spike and deform it as occurs at the endplate region of the muscle fibre (FATT and KATZ, 1951; DEL CASTILLO and KATZ, 1954b). The latter effect is essentially a poising of the membrane potential at the endplate region about the level of a nonselective diffusion potential (DEL CASTILLO and KATZ, 1954b), requiring a concentration of postsynaptic membrane which is not available in the eel electroplaque.[37a]

VII. FURTHER DETAILS OF THE ELECTRICALLY EXCITABLE RESPONSE

The foregoing account of the discharge of single units of the eel electric organ has now made a full swing from the spike of the denervated, electrically excitable membrane to that elicited by synaptic excitation in the normal cell. As pointed out earlier, the latter spike in no way differs from the former. However, several factors provide some additional information on the electrically excitable response of the eel electroplaque which are not as easily observed in other cells. The slow conduction rate of the spike in the electroplaque indicates that the characteristic length of the membrane is very short (KATZ, 1939; HODGKIN and RUSHTON, 1946) in comparison with the length of the cell. This is borne out by the absence of electrotonic pickup of activity about 2 mm from a still-quiescent region (Fig. 13, etc.). The size of the cell also easily permits insertion of several microelectrodes to study experimentally[38] simultaneous electrical effects which might occur in the cell in its normal physiological state (ALTAMIRANO *et al.*, 1955a), or under the special conditions of drug action (ALTAMIRANO *et al.*, 1955b). These particular advantages of the eel electroplaque, as experimental material, have disclosed that the electrically excitable membrane responds electrogenically in two modes, the all-or-nothing, and the graded from minimal to the maximal e.m.f. of which the cell is capable (ALTAMIRANO *et al.*, 1955a; cf. also ALTAMIRANO and GRUNDFEST, 1954; GRUNDFEST, 1956a).

flows and minimal responses. The persistence of such responses when the presynaptic terminals are presumably fully depolarized (DEL CASTILLO and KATZ, 1955b) would seem to indicate the possibility of this condition (cf. also LILEY, 1956).

[37a] LETTVIN *et al.* (1956) have shown with an ingenious technique that the antidromically evoked spike of cat motoneurons is reduced in amplitude by the simultaneous development of a p.s.p. in the cell. The magnitude and time course of this reduction presents an inverted image of the p.s.p. itself. This may be interpreted as an inversion of the p.s.p. such as would be expected during the reversed polarization of the overshoot of the spike (DEL CASTILLO and KATZ, 1954b). This finding therefore constitutes a new demonstration that the p.s.p. of cat motoneurons develops in membrane which is not electrically excitable.

[38] Other physiological and physical factors such as the possibility for delivering graded stimuli directly or through neural pathways, and the properties of a plane-surface excitable membrane, were discussed briefly by ALTAMIRANO *et al.* (1955a).

VII. 1. *Graded response during refractoriness*

As the electroplaque recovers from absolute refractoriness, a strongly supraliminal stimulus at first elicits a small response localized to the stimulating site. The response grows in amplitude rapidly (Fig. 27; cf. also Fig. 16), but remains decrementally propagated to more distant loci even though it develops an overshoot. Transition from decremental propagation to the all-or-none condition of the decrementlessly propagated spike occurs suddenly, usually with no observable change

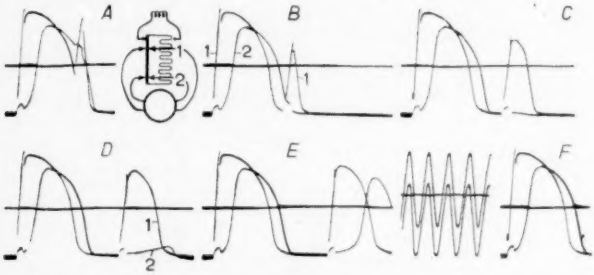


Fig. 27. Graded response in eel electroplaque during early relative refractoriness. A strong direct stimulus was used to test responsiveness of the cell after a previous conditioning stimulation. The cell was in absolute refractoriness during the conditioning spike (*A*, *B*). Later (*C*) a response with overshoot occurred at the recording site (1) near the stimulating locus, but no activity developed at the distal site (2). Somewhat later still (*D*) the stimulus evoked a large response at 1, but very small depolarization at 2. Propagation developed later in recovery (*E*), but still in relative refractoriness as denoted by the smaller delayed spike in comparison with the control (*F*) (cf. also Fig. 16). The transition from graded, decrementally propagated responsiveness in *D* to the decrementless, all-or-nothing variety (*E*) occurred with almost no change in the amplitude of the response at site 1. Calibrations 100 mV and msec. Distances 0·59 and 2·13 mm. (From ALTAMIRANO *et al.*, 1955a.)

in the form or amplitude of the response. The same sequence of events also is disclosed when the testing stimulus is a maximal neural volley following a directly elicited conditioning spike (Fig. 16).

VII. 2. *Graded responsiveness induced by chemical treatment*

The disclosure of graded responsiveness gains theoretical importance from the behaviour of cells poisoned with the nondepolarizing synaptic blocking agents (Table 1, Figs. 28 and 29). When the neurally evoked response is no longer elicited, the cell remains electrically excitable (DU BOIS-REYMOND, 1881, p. 202; CHAGAS and ALBE-FESSARD, 1954; ALTAMIRANO *et al.*, 1955b). However, the character of the response alters (ALTAMIRANO *et al.*, 1955b), becoming graded and decrementally propagated (Fig. 28). Strong stimuli evoke a response which in form and amplitude is identical, or nearly so, with the normal spike. Spread of increasingly strong stimuli along the excitable plane membrane

elicits maximal activity at successively more distant regions, eventually activating the whole cell. The appearance of these maximal responses recorded simultaneously at two sites is then identical with that of the normal spikes (Fig. 28).

Nor is it necessary to employ strong stimuli to elicit maximal responses (Fig. 29), since direct excitation of the eel electroplaque leaves behind a period of enhanced excitability lasting about 300 msec

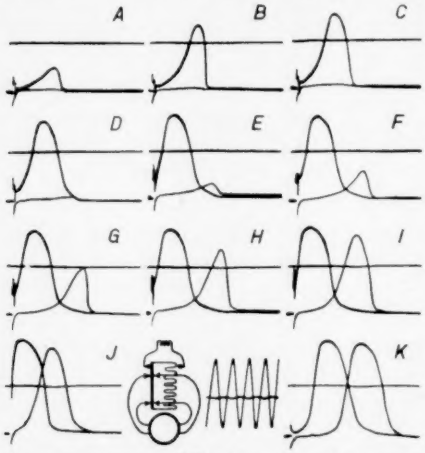


Fig. 28. Graded responsiveness following chemical treatment of the eel electroplaque. The initial responses of the cell to a direct stimulus are seen in *K*. After poisoning the cell with 1 mg/ml eserine the stimulus evoked only a small response at the proximal site, nothing at the distal site. Increasing strength of stimulation increased this response (*B–D*), still with no distal activity. The latter began to develop with stronger stimuli, showing the full range of gradation (*E–J*), but the response at the proximal site remained essentially unchanged, although arising with briefer latencies. The responses in *J* are almost of the same amplitude as are the spikes of the unpoisoned cell *K*. The resting potential remained unchanged throughout. Calibration 100 mV and msec. Distances 1 mm and 1·85 mm. (From ALTAMIRANO *et al.*, 1955b.)

(ALTAMIRANO *et al.*, 1953). Stimulation of the poisoned cell at 5/sec with slightly subthreshold stimuli then elicits responses increasing gradually from a small amplitude to activity which approximates the normal spike (ALTAMIRANO *et al.*, 1955b).

VII. 3. *Nature of graded responsiveness*

Increase of local response during relative refractoriness was observed by HODGKIN (1938) and appeared to be satisfactorily accounted for by the ionic theory (HODGKIN and HUXLEY, 1952). It was suggested that increased potassium conductance during the recovery period and persistent sodium inactivation should result in a response in which the inward flow of sodium does not sufficiently exceed the outward potassium flow to initiate regenerative action or propagation by local circuit

excitation. This explanation may be adequate for the squid giant axon, since during relative refractoriness the membrane potential is larger than the resting value and is consonant with high potassium outflow.[39] However, the response of the eel electroplaque does not exhibit a hyperpolarizing phase (e.g. Figs. 28, 29). Furthermore, the ability of the recovering electroplaque to develop a nearly maximal, but graded, decrementally propagated response implies that sodium

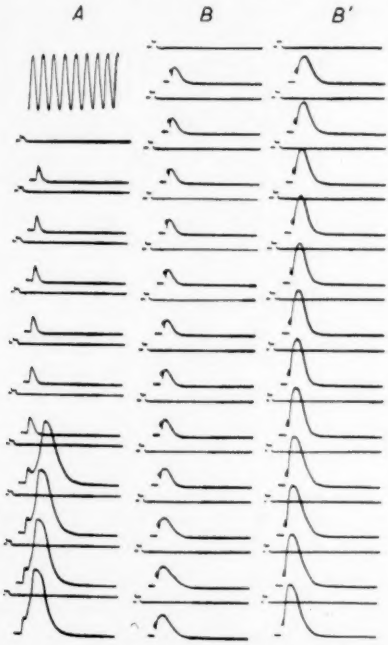


Fig. 29. All-or-nothing and graded responses of eel electroplaques elicited by latent addition of excitatory effects. *A*. Consecutive records (from above down) at 5/sec using a weak, direct stimulus. Two traces are in each record, the upper and longer being the zero reference base line on which is also a monitor of the stimulus. The trace below, deflected by the resting potential, is that of the microelectrode lead. The repetition of weak stimuli increased excitability of the cell by latent addition, which lasts about 300 msec. An all-or-nothing spike arose suddenly. *B–B′*: A similar sequence but 88 min after treating the cell with 500 μg/ml eserine. The stimulus was somewhat stronger, but as before was maintained at constant amplitude. The graded response visible on the first record of the series increased at first slowly and then more rapidly, showing the range of gradation up to a spike-like response (although smaller than the latter) at the termination of the series. The resting potential was unchanged during the entire experiment. Calibration 100 mV and msec.
(From ALTAMIRANO *et al.*, 1955b.)

[39] This holds only during the early phase of the hyperpolarization of the squid giant axon. The later portion is associated with increased membrane resistance and therefore with a lower K^+ outflow than in the resting state (SHANES *et al.*, 1953), and is probably a consequence of the hyperpolarization itself (AMATNIEK *et al.*, to be published).

influx at this stage is about as large as in the normal cell and must temporarily far exceed the potassium outflow. Also, the gradedly responsive poisoned cells do not undergo depolarization which is the presumed cause of heightened sodium inactivation.

For these reasons, one or more other membrane processes must be postulated in addition to those of the ionic theory. Their existence also in other cells, including the squid and other giant axons, has been indicated by the finding of spike-like, but decrementally propagated response (GRUNDFEST and KAO, 1955; KAO and GRUNDFEST, 1955; GRUNDFEST, 1956b). The nature and kinetics of these events are not as yet known. The regenerative process of the all-or-nothing response appears to be broken temporarily during refractoriness, permanently when the electroplaque or axon is poisoned with the non-depolarizing drugs. This suggests that regenerative involvement of sodium "carrier" or "valving" units of the electrically excitable membrane is impaired, these becoming not inactivated, but less easily responsive, to the stimulus of membrane depolarization. On the basis of this suggestion, the elements of the normal cell membrane have nearly identical thresholds for the triggering of their transducer action. During recovery from refractoriness these thresholds might have different recovery kinetics. With different sensitivities to the poisons a similar spread of thresholds would develop (GRUNDFEST, 1956b,d).[39a]

The above suggestion, though qualitative at the present time, is equivalent to the assumption that the amplification factor of the membrane transducer is high in the normal case, but low in the gradedly responsive membrane. An electrical analogue of this condition can be obtained in monostable electronic circuits. Operating at low loop gain, and with a short time constant of coupling, the circuit responds in a fully graded manner to increasing trigger pulses (Fig. 30).

VII. 4. *Graded responsiveness in other electrically excitable cells*

The analogy, of course, does not provide a clue to the specific nature of the membrane events which cause graded responsiveness in living tissues. The reality of the phenomenon and its existence as one form of

[39a] This interpretation, that graded responsiveness is caused by an altered distribution of the thresholds of the population of electrogenic units (valves) of the electrically excitable membrane, accounts for an interesting recent finding (ALTAMIRANO, 1956a). Although a poisoned eel electroplaque responds gradedly to a punctate electrical stimuli, as in Figs. 28 and 29, its response appears to be all-or-none under conditions of "space clamping" (i.e. the entire membrane is stimulated at once by massive electrodes). Everywhere in the membrane all the low-threshold elements of the electrogenic population should then be excited simultaneously. This should produce sufficient general depolarization to trigger the response of the next most excitable elements, etc., leading to the spikelike appearance of the activity. It may be noted, incidentally, that each electrogenic "valve" or "pore" of the electrically excitable membrane appears to switch temporarily under the trigger of depolarization from sensing the "potassium potential"

normal electrically excitable responsiveness appears to be well established. "Functional refractoriness" of vertebrate axons has been studied extensively (ROSENBLUETH *et al.*, 1949). This stage is associated with graded responsiveness in the squid giant axon (GRUNDFEST and KAO, 1955; KAO and GRUNDFEST, 1955), in earthworm and crayfish giant axons (KAO and GRUNDFEST, 1956, and to be published), and in

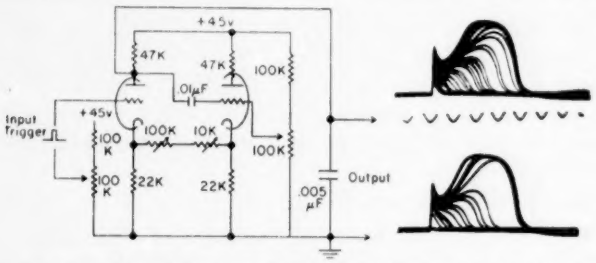


Fig. 30. An electronic analogue of graded responsiveness. *Left*: A monostable circuit was modified to permit variation of the overall amplification, by the resistors between the cathodes of the 12AU7 tube. The output capacitor increased the rise time of the output pulse and shaped it to resemble somewhat the spike of an electroplaque. The lower record on the right, a series of multiple responses under conditions when a "local response" developed on increasing the trigger pulse and then erupted into a "spike." *Upper set*: Decreasing the overall loop gain of the circuit produced output pulses which were completely graded in amplitude. Time in milliseconds.

cardiac fibres (HOFFMAN and KAO, 1956). The occurrence of "abortive" spikes was noted by HODGKIN (1938) in single axons, and is frequently seen in the squid giant fibre, and elsewhere (cf. Fig. 14), including the partially curarized muscle fibre (ECCLES and O'CONNOR, 1941). Many invertebrate muscle fibres appear to respond only gradedly to direct stimulation (e.g. HAGIWARA and WATANABE, 1954; FURSHPAN, 1955; cf. ALTAMIRANO *et al.*, 1955a). Some mammalian smooth muscle is similarly responsive.[40]

to reflecting approximately the "sodium potential." Thus, an assembly of unitary, all-or-none responses can nevertheless manifest a graded response by virtue of a distribution of the population with respect to thresholds. Similar conversion of all-or-none unitary responses into quantitatively graded ensembles occur in other systems as well (e.g. hemolysis of red cells).

[40] Graded responsiveness in these probably was the condition which led to the view that they were not electrically excitable (cf. ROSENBLUETH, 1950). This is particularly evident in the behaviour of uterine muscle. During the anoestrous phase only local responses are observed (BOZLER, 1941), whereas during the oestrous the responses are propagated and presumably all-or-nothing. The two conditions can be brought on at will by hormonal treatment (BOZLER, 1941; CSAPO, 1954). Poisoning striated muscle with procaine (which in the eel electroplaque acts similarly) causes loss of propagated responsiveness, but leaves the muscle capable of contractions localized to the stimulating cathode (STEN-KNUDSEN, 1954). Alteration of responsiveness in nerves from the all-or-nothing to the gradedvariety was probably responsible for the earlier conclusion (e.g. BULLOCK *et al.*, 1947) that deprivation of cholinesterase eliminated their electrogenic responsiveness. As in the eel electroplaque (ALTAMIRANO *et al.*, 1955b) treatment

VIII. Participation of the Several Electrogenic Components in the Reflex Discharge

The information available on central control mechanisms of electric fishes does not, as yet, permit detailed analysis of their actions although, as described earlier, some problems relating to reflex discharges have been satisfactorily explained. It is not known, for example, whether the neuronal complexes of the fish central nervous system are endowed with inhibitory as well as excitatory processes, although the presence of the former is to be suspected from indirect evidence (cf. footnote 7, p. 10). The frog nervous system is so endowed, and this case appears to have been the earliest demonstration of central inhibitory systems (Sechenov, 1863).

It is also unknown whether the electric organs have regulatory feedback mechanisms. Indeed, the absence of mechanical response in electric organs would make it difficult to envisage the types of feedback regulation so exquisitely developed for control of muscular activity (Kuffler and Hunt, 1950; cf. Granit, 1955). Yet the extraordinarily stable frequencies of the constantly emitting fishes (Fig. 9) strongly indicate that some type of control must be operating, and furthermore the contrast between these electric fishes and the normally quiescent must be ascribable to specific mechanisms. The properties of the synaptically and electrically excitable electrogenic membranes, their proportions in various electric organs, and the amounts of innervation of the different electroplaques, can account for some of the differences in the types of responses reflexly initiated in various electric organs.

VIII. 1. *Electrophorus*

The role of local electrogenic mechanisms may be expected to be somewhat more complicated in the reflex activity of the individual eel electroplaque than in the reflexly initiated behaviour of the electroplaque's relative, the skeletal muscle fibre. This derives from their different modes of innervation. The muscle fibre is frequently innervated at several loci (Katz and Kuffler, 1941; Hunt and Kuffler, 1954), but each endplate is presumably always supplied by one nerve fibre. It is conceivable that a single impulse in one or the other innervating axon does not fire a muscle fibre, but these cases must be rather rare since they are not noted in the literature.[41] On the other hand,

of squid giant axon with eserine leaves it gradedly responsive (Grundfest and Kao, 1955; Kao and Grundfest, 1955, and unpublished).

[41] It would be rather difficult to tell except in special preparations. Some of the diffusely and multiply innervated, gradedly responsive invertebrate fibres (cf. Fatt and Katz, 1953) or vertebrate fibres (cf. Burke and Ginsborg, 1955) may be expected to have nerve supplies of different synaptic potency.

the eel electroplaque does not seem to discharge by the impulse of any one of its supplying nerve fibres, since small, subthreshold p.s.p.'s are seen with weak neural stimulation (Figs. 14, 26). Indeed, some nerve trunks do not discharge the cell even when they are maximally stimulated (Figs. 22, 25). This also occurs in the whole animal, particularly in the main organ which, as noted earlier, discharges weakly to an initial stimulus while subsequent responses become stronger or maximal through facilitation. The reflex discharge of the intact fish shows this effect (Fig. 8). The responses are initiated with a small potential, probably composed of the activity of a small number of cells of the discharge zone (Fig. 22), but to which the p.s.p.'s of the entire organ must also contribute (ALTAMIRANO *et al.*, 1953). The subsequent responses are maximal, within the large error of the observations.

VIII. 2. *Malapterurus and Torpedo*

The reflex discharges of these fish seem to be devoid of initial gradations (Fig. 8). Innervation of each electroplaque by a single nerve fibre, and of all the cells of the half-organ by the same fibre, makes the situation in *Malapterurus* somewhat like that in a motor unit of skeletal muscle. As noted earlier, it is likely that graded responses would be obtained from *Torpedo* electroplaques if their individual nerve fibres were separately stimulated. However, the densely distributed multiple innervation of the cells (Figs. 1 and 6) probably assures a maximal discharge of the organ in the intact animal, despite the likelihood that this response is a p.s.p., not a spike.

VIII. 3. *Repetitively discharging organs*

While the details of their activity are not as yet known, the high rate of discharge in some of the fishes (Fig. 9), its regularity, and central control, is worthy of analysis in terms of the underlying electrogenic mechanisms. In the eight species studied thus far in this laboratory (COATES *et al.*, 1954; unpublished work with MAURO and AMATNIEK), the responses of the whole animal are remarkably constant in amplitude. Since the number of electroplaques is small, as indicated by the small total voltage of the discharge and by preliminary anatomical studies, it is likely that all, or nearly all of the cells participate in each discharge. It seems probable that the repetitively discharging cells are capable of this behaviour because of a low rate of sodium inactivation and the associated "slow adaptation."[42]

Examples of different degrees of "adaptation" studied in correlation with the membrane potentials are now available. The single autonomic

[42] As used here, "sodium inactivation" perhaps includes more than was intended by HODGKIN and HUXLEY (1952), since it envisages effects sometimes developing over very long periods of time in comparison with the time scale in their experiments. It is

ganglion cells of the rabbit cannot follow with spikes synaptic activations at rates above about 80/sec (ECCLES, 1955). Even at this rate, the spikes are strongly depressed (*loc. cit.* Fig. 8). At shorter intervals between the synaptic stimuli an initial spike of the cell is followed by a second depressed discharge and then by small modulations, perhaps of graded responses (as in Fig. 17) superimposed on a depolarization of about 40 mV developed by summation of the p.s.p.'s. Except for the time scale and the number of discharges, the behaviour of the rabbit ganglion cell resembles that of a slowly adapting crayfish stretch receptor depolarized by successively stronger stretch stimuli (EYZAGUIRRE and KUFFLER, 1955a, Fig. 9). In both cases it is likely that the same mechanism is operative, that of sodium inactivation. In the ganglion cell, this process strongly developed by depolarization would operate to block quickly a repetitive discharge.[43] A slower onset of inactivation for the same degree of depolarization would permit the crayfish stretch receptor to discharge continuously for a considerable time. Differences in "adaptation" of the gamut of sensory cells studied for this phenomenon may be similarly explained, as an effect upon electrically excitable membrane having different kinetics of inactivation.[44]

Depolarized by a sustained applied stimulus, the abominal ganglion cell of *Aplysia* also exhibits repetitive activity (TAUC, 1955). At the end of each spike the membrane potential temporarily undershoots the depolarized level, descending toward the resting potential, as is also

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used in preference to "accommodation" for two reasons. The attempt to attach a more specific mechanism to the phenomenon of accommodation should provide a challenge for future experiments. Furthermore, "adaptation" and "accommodation" are applied to many other phenomena, including those presumed to occur in sense organs. The emphasis here is that the phenomenon basically occurs in the electrically excitable action generator. The membrane which is not electrically excitable, whether in the synaptic structures or in the sense organs (cf. GRUNDFEST, 1956b,d) probably is not subject to "accommodation" or "adaptation." Thus, the depolarized eel electroplaque still generates the p.s.p. when it becomes electrically inexcitable (Fig. 17). A low critical depolarization for discharging the spike may serve as a contributory factor to a slowed rate of "accommodation" by sodium inactivation, the discharging spikes tending to keep the membrane potential at a less depolarized level (cf. EYZAGUIRRE and KUFFLER, 1955a; DEL CASTILLO and KATZ, 1955c; and below).

[43] The axons of cat sympathetic ganglia can discharge at a high rate to repetitive stimulation (GRUNDFEST and GASSER, 1938), and it is probable that the fibres of the rabbit can do so also. Although repetitive direct stimulation presents a somewhat different situation from that of sustained depolarization by summated p.s.p.'s, in the cat the negative after potentials summate during a train of responses to cause steady depolarization. C fibres also have slow "accommodation." These various facts indicate the possibility that the cells and axons may have different kinetics of inactivation. The effect observed by R. M. ECCLES (1955; Fig. 8) is strictly that of classical Wedensky inhibition (cf. GRUNDFEST, 1956d).

[44] It is not intended to rule out other mechanisms for adaptation: (*a*) In the rapidly adapting crayfish stretch receptor, the depolarization of the soma to a sustained stretch of the dendrites steadily decreases (EYZAGUIRRE and KUFFLER, 1955a). When the level

the case for the crayfish stretch receptor (EYZAGUIRRE and KUFFLER, 1955a,b) and the frog muscle fibre (cf. THESLEFF, 1955a; Fig. 7). The membrane potential then returns at a slow rate which is characteristic of the long time constant of the *Aplysia* cell membrane, and a new spike develops. However, continued depolarization of the membrane decreases the amplitudes of the spikes and their rate of onset, both phenomena which can be accounted for by inactivation. An example of tissue which seems to have no inactivation is probably provided by the pacemakers of cardiac muscle. Under the stimulus of a steady depolarization, derived from an as yet unknown source, the membrane undergoes rhythmic discharges which also involve a temporary undershoot of membrane potential (cf. DEL CASTILLO and KATZ, 1955c; HUTTER and TRAUTWEIN, 1955).[45]

Repetitive discharges sustained for various periods of time are also seen in various types of axons, and the frequency of discharge may be correlated with the time constants of their membranes, including the rate of growth of the local response (cf. HODGKIN, 1948) as well as with

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falls below that required to fire the electrically excitable membrane, the cell ceases to discharge spikes. Whether the effect is due to a decrease of the generator potential at the mechano-transducer terminals (e.g. by a mechanism of "differentiation" such as those discussed by KATZ, 1950, for the muscle spindle) or is secondary (e.g. by a change in the membrane properties of the dendritic stem resulting in a smaller length constant for electrotonic transmission) is not known. (*b*) Continued response to a sustained stimulus might also deplete the stock of transmitter from a specialized receptor cell, as apparently happens in repetitive neuro-muscular transmission. However, the slowly adapting crayfish stretch receptor is an example with directly observed electrical properties which do not lend themselves to either of the above alternative explanations, and this is probably also the case with the frog spindle (KATZ, 1950).

A query may be raised here with regard to many of the cells classified as sense organs (cf. also, WEDDELL *et al.*, 1955). Are they in reality specialized transducers, transmitting in turn to their nerve supply, or are these cells perhaps accessory to sensory membrane which resides in the terminals of the nerve fibres? Free nerve terminals subserving sensory functions are numerous. The sensitivity and variety of sense perceptions of the terminals in the olfactory epithelium is remarkable (cf. OTTOSON, 1956). The olfactory membrane, electrogenic in response to chemical stimuli, does not become activated by antidromic invasion (OTTOSON, 1956). It therefore appears to be electrically inexcitable. A nerve terminal, apparently thinly myelinated and modified from the rest of the fibre extends into the Pacinian corpuscle (QUILLIAM and SATO, 1955). This terminal might itself be the sense organ, surrounded by an inert although conspicuous structure, perhaps having a function in the mechano-reception analogous to that of the outer ear in hearing.

[45] The single spikes of crayfish stretch receptor (EYZAGUIRRE and KUFFLER, 1955a) or of cardiac muscle (WEIDMANN, 1956) usually do not have an undershoot. TAUC (1955) finds the same for *Aplysia* ganglion cells, but ARVANATAKI and CHALAZONITES (1955) show spikes with undershoot. The records of the latter, however, are primarily concerned with repetitively, usually spontaneously discharging cells, which may be expected to have the repolarizing undershoot. The development of this phase in the response probably indicates that heightened potassium conductance occurs which can temporarily recharge the membrane capacity to a level near that of the potassium battery (HODGKIN and HUXLEY, 1952). The bearing of this on the interpretation of the repetitive discharges of electric fishes will be mentioned below.

the effects of inactivation, which in addition would determine the duration of the discharge. Axons which do not normally exhibit repetitive discharge can be made to do so under a variety of stimuli (ADRIAN, 1930; LEHMANN, 1937a,b; ARVANITAKI, 1939; BRINK *et al.*, 1946). In the squid axon, which belongs to the above category, repetitive discharge to applied depolarizing currents is rare. However, discharges at up to 300/sec sustained for 5 or 10 sec are frequently seen during depolarization of the axon by KCl. As the membrane potential decreases, the spikes become smaller and eventually are blocked. Reversal of the membrane potential change, by washing out the KCl, may initiate a new burst of repetitive activity until the membrane potential rises toward its normal value.

Since the continuously repetitive discharge of the electric organs is centrally controlled, it may be supposed that not only the electroplaques, but also the controlling neuronal complex has slow "accommodation," or as has been suggested in the foregoing, a low rate of sodium inactivation. However, the ability to maintain indefinitely central nervous activity at rates as high as 1000/sec (Fig. 9) is not in keeping with the general behaviour of neuronal synaptic systems. At a constant temperature, the frequency of the responses is remarkably constant to about 1 per cent. It is also independent of the movement of the fish, at least in most of the species examined in this laboratory, even when provoked by strong electrical stimuli (Fig. 9). These properties are difficult to envisage as involving discharges of electroplaques one-to-one by centrally initiated neural volleys. The facts therefore indicate either special properties or a subsidiary mechanism. A possible mechanism (at present being sought for experimentally) would involve neuronal discharges at a slow rate and effector activity at a much higher rate, which could be provided by the coupling of membranes with different electrogenic properties. A slow rate of neuronal discharge could maintain depolarization by summation of p.s.p.'s, and this, stimulating poorly inactivable electrically excitable membrane, could elicit spikes at a high rate. Decrease or increase of the number of neural impulses could lower or raise the level of steady depolarization and thereby modulate the rate of spike discharges. Sustained depolarization by p.s.p.'s accounts for the repetitive discharges at up to 300/sec of the axono-axonally innervated segmental giant axons of earthworm and crayfish after a single stimulus (KAO and GRUNDFEST, 1956; and unpublished). Repetitive activity at high rates in FLECHSIG's tract (GRUNDFEST and CAMPBELL, 1942; LLOYD and MACINTYRE, 1950), in the Renshaw cells of the cat spinal cord (ECCLES *et al.*, 1954), and in other spinal interneurones (FRANK and FUORTES, 1956), may have a similar origin, sustained depolarization of the postsynaptic membrane, reacting upon electrically excitable

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membrane which is not rapidly inactivated by the depolarization. Different time constants of the membrane, critical firing levels, and degrees of sustained depolarization, could account for the variety of rates observed in the spinal neurons as well as the remarkable constancy in the pattern of repetition found in some (cf. FRANK and FUORTES, 1956, Fig. 14).[46]

In some of the continuously emitting fishes the discharges recorded from the intact animal appear as a predominantly monophasic pulse, but with a small reverse component. The reversed potential, which is not ascribable to organ complexity as it is in *S. albifrons* (Fig. 9), could be an undershoot following the spike elicited on a sustained depolarization, such as occurs in *Aplysia* ganglion cells, crayfish stretch receptors, and the cardiac pacemaker. The spike of the eel electroplaque does not have this component (Figs. 11, 13), nor do the reflex discharges of the intact, normally quiescent fish show this phase (Fig. 8).

IX. The Role of Different Electrogenic Membrane Components in Pharmacologically Altered Responses

CLAUDE BERNARD's discovery of the curare effect on synaptic transmission in muscle has since then impelled physiologists and pharmacologists to consider transmission as a peculiarly labile phenomenon, chemically mediated.[47] Nevertheless, the manifestations of drug actions have presented a number of puzzling discrepancies which have often induced doubt in the significance of pharmacological findings (the writer expresses here a *mea culpa*), and the minimizing of its evidence (cf. Discussion, p. 434, BOVET, 1951). The pioneering work relating actions of synaptic drugs to their effects on electrogenic mechanisms is relatively recent (ECCLES *et al.*, 1941; KUFFLER, 1942;

[46] Other mechanisms of frequency multiplication are also possible in various cases. The wing muscles of the bee contract more than once for each nerve impulse, and this appears to be caused by mechanical restimulation of the muscle during the wing beat (BOETTIGER, 1956). These muscles might therefore have mechano-transducer membranes (GRUNDFEST, 1956b). The findings that the electrical response of muscle is increased by stretch (KUFFLER, 1952; RALSTON and LIBET, 1953), that calcium-deficient muscles respond with repetitive activity to stretch or pressure (ADRIAN and GELFAN, 1933) and proportionally to the applied tension (BÜLBRING, 1955) suggest the possibility that vertebrate muscle is also endowed with mechano-transducer membrane (GRUNDFEST, 1956b). HUTTER and TRAUTWEIN (1956), on the other hand, have presented evidence indicating that the increased response of stretched partially curarized frog muscle is due to the effect of the tension on the prejunctional nerve terminals. This would indicate (alternatively or concurrently) that these nerve endings also act as mechano-transducers, more transmitter being released when they are in tension (cf. also BÜLBRING, 1956).

[47] While many modern-day electrophysiologists have been reluctant to accept the latter, DALE (1954) has pointed out that DU BOIS-REYMOND suggested, and even definitely favoured, the idea that transmission is by a chemical stimulant, rather than by an electrical process. Earlier in the present review it was noted that BERNSTEIN, GOTCH, and GARTEN had envisaged a difference between neural and electrical excitation of muscle and electroplaques.

ECCLES, 1944). Current pharmacological research has also been concerned with these aspects, and has provided a more detailed background of data and some interpretations (cf. PATON, 1954; ZAIMIS, 1954) which help to explain the pharmacology of electric organs. On the other hand, recognition of differently responsive electrogenic mechanisms gained by the study of muscle and electric organs can also serve to illuminate the general theory of the synaptic effects of drugs.

The innervated eel electroplaque, possessed of differently electrogenic membrane components, has provided a relatively comprehensive study at the cellular level of the actions exerted by ten synaptic agents (ALTAMIRANO *et al.*, 1955b). The actions studied were those on synaptic excitability and facilitation (Fig. 23), on resting potential, spike and p.s.p. (Figs. 17, 20, 28, 29), as well as on some secondary (Figs. 28, 29) and antagonistic effects. Other work (CHAGAS and ALBE-FESSARD, 1954), although less detailed in scope, is in close agreement and has furnished some data on two additional substances (Table 1), as well as confirmation of the interpretations.

While the data available on the eel electroplaque can be incorporated into general concepts of synaptic pharmacology, the preparation used, an isolated, relatively simple synaptic system, must necessarily fail to provide the variation and interactions which the study of more complex systems or of the whole organism supplies. The latter effects are often confusingly complicated, although they are of paramount clinical importance and sometimes contribute to fundamental knowledge of mechanisms. Some of the factors which are involved in these effects can be envisaged and explained in terms of the data gained on the simpler synaptic preparations, sufficiently so as to encourage the view that other complexities bear not against the "principle, but only its perfection in detail" as DALE (1954, p. 10) has said in another context.

IX. 1. *Classification of the action of synaptic agents*

The broad division of synaptically acting drugs into depolarizing and non-depolarizing (competitive antagonist) substances (cf. PATON, 1954; ZAIMIS, 1954) is borne out in the studies on the eel electroplaque preparation (Table 1). Nevertheless the terminology does not precisely describe the drug actions, and this appears to confuse their interpretation. The "depolarizing" substances act as such on the eel electroplaque and on muscle fibres, both of which have depolarizing p.s.p. On the other hand, acetylcholine hyperpolarizes (or repolarizes, KUFFLER and EYZAGUIRRE, 1955) cardiac muscle (BURGEN and TERROUX, 1953; HOFFMAN and SUCKLING, 1953; DEL CASTILLO and KATZ, 1955c; HUTTER and TRAUTWEIN, 1955). The cholinergic nerve does the same at the pacemaker cells (DEL CASTILLO and KATZ, 1955c;

HUTTER and TRAUTWEIN, 1955). These cells, being provided with a hyperpolarizing p.s.p. therefore respond to the "depolarizing" drug with hyperpolarization. In the remarkably illustrative example of the cat salivary glands (LUNDBERG, 1955a) some cells respond only with hyperpolarization to stimulation of both the cholinergic and adrenergic nerves. These cells also respond in the same way to acetylcholine, adrenaline, and pilocarpine.

Therefore it may be concluded that the "depolarizing" drugs activate the synaptically excitable membrane component and that the electrogenic effect is determined not by the drug, but by the type of the excited membrane. These agents should be designated more correctly as "synapse activating" substances. The "non-depolarizing" compounds, in contrast, appear to block the responsiveness of the postsynaptic membrane (Fig. 20) and may therefore be symmetrically designated as "synapse inactivating" substances.

IX. 2. *Overt effects in terms of electrogenic mechanisms*

The effects of synaptic agents on the entire excitable system will depend on the type of electrogenic reaction which the substances evoke and on the properties of the excitable membranes. The synaptic activator, succinylcholine, depolarizes the endplate, and this electrogenesis, at first, stimulates the electrically excitable component of the muscle fibre. This is manifested as "fasciculation."[48] Subsequently, the electrically excitable membrane in the region of the endplate (and in some cases more extensively) becomes unresponsive, very probably through the mechanism of sodium inactivation. "Curarization" then develops.[48a] In muscles with a low rate of inactivation, the response would be a contracture. On the other hand, the hyperpolarizing action of acetylcholine on the cardiac pacemaker cells represses their rhythmic depolarization, slowing or stopping the cardiac rhythm. Cases may be conceived of in which the same drug activates both depolarizing and

[48] The term is inappropriate for this condition, which is more justly characterized as fibrillation *en masse*. The mechanism is that of excitation of each muscle fibre on its own, rather than the obligatorily synchronized discharge of the entire motor unit by the innervating motor fibre which is the proper meaning of fasciculation. The misnaming derives from the character of the overt response—a massive, tetanic, and generalized contraction.

[48a] "Curarization," like "fasciculation," is inappropriate in the context. The term should be reserved to describe the action of truly curarimimetic drugs: blockade by inactivation of synaptic electrogenesis without depolarization. Grossly, this action is denoted by one or several criteria: persistence of direct excitability, absence of contracture, competitive abolition of blockade by "depolarizing" drugs, enhancement of the effect by other synapse inactivating drugs. When responses are blocked by persistent depolarizing synaptic electrogenesis through the action of synapse activating drugs the effect should be termed "depolarizing blockade." The overt criteria of the mechanism are the inverse of those listed above. Chemical activation of inhibitory electrogenesis might also produce an appearance of "curarization" which can be more precisely described as "hyperpolarizing (or inhibitory) blockade."

hyperpolarizing postsynaptic membrane. The net effect on the electrically excitable membrane would then be the resultant of the balance of electrical change and of the possible predominance of one type of electrogenesis over the other in specific regions of a cell. The overt effects might be as striking as is the inhibition of reflex activity by the essentially similar, but temporally determined actions of neural volleys (Brock *et al.*, 1952; Eccles *et al.*, 1954), or they might degenerate to the relatively insignificant proportions caused by mild hyperpolarization of the conductile membrane (cf. Fig. 19).[49]

Thus, the synapse activating substances, exerting their primary effects upon the different kinds of electrogenesis of the postsynaptic membrane, may be expected to evoke different overt responses. These are in general a consequence of the secondary action of the synaptic electrogenesis on the electrically excitable membrane and will depend also on the specific properties of the latter. As was indicated in the preceding sections, it is likely that in various electrically excitable tissues the kinetics of the several ionic processes differ. These variations superposed on those of the synaptic electrogenesis create the possibility of a wide range of overt responses to the activating drugs.[50]

Another type of situation may develop in systems which are only synaptically electrogenic. For example, some muscle fibres do not have an electrically excitable membrane (e.g. Burke and Ginsborg, 1955). The long-term depolarization of their diffuse endplates probably is not affected by a sodium-inactivation process, and their mechanical responses are not dependent on a spike. It may be expected therefore that these fibres would react with a contracture to acetylcholine or its congeners. Similarly, the injection of acetylcholine into the *Torpedo* electric organ yields a prolonged electrical discharge (Feldberg and Fessard, 1942) which does not appear to be an accumulation of individual discharges.

The nondepolarizing, synapse inactivating agents block the electrogenesis of the postsynaptic membrane and thereby also exert specific types of overt response. These compounds probably act on the hyperpolarizing synapses in the same way they do on the depolarizing,

[49] Perhaps this might help to explain the overtly manifested relative insensitivity of motoneurons to synaptic drugs (cf. Eccles *et al.*, 1956).

[50] Zaimis (1954, p. 57) has remarked that, while the interpretation of results obtained with a "competitive substance is relatively straightforward . . . the interpretation of the results obtained from different species and from different muscles (with 'depolarizing' substances) needs great care." The kinetics of the different ionic processes could be altered merely by changing the triggering thresholds within the populations of electrogenic units of the membrane, as discussed in Section VII, 3. In the synaptic membrane component the thresholds would be in terms of chemical sensitivity, not of membrane potential. Given the probability that these populations are heterogeneous with respect to valving sodium, potassium, chloride, and perhaps other ions, complexity of overt phenomena is to be expected (cf. Section IX, 4).

without changing the resting potential of the cell. Thus, the muscle fibre, its electrically excitable membrane no longer subjected to depolarizing synaptic electrogenesis, is not synaptically excitable. However, the absence of the synaptic depolarization, such as is caused by the activating substances, leaves the electrically excitable membrane responsive to direct stimulation. The heart becomes unresponsive to vagal stimuli after curarization, but its myogenic electrically excitable rhythmicity is unaltered.[51] On the other hand, the *Torpedo* electric organ becomes inexcitable after curarization, and this effect is to be expected in cells in which only synaptically excitable membrane is present. A similar case is that of cortical dendrites (GRUNDFEST and PURPURA, 1956). Their electrical response is rapidly and reversibly abolished upon curarization. It is therefore inferred that these extensions of electrically excitable cortical cells are possessed only of postsynaptic electrogenic membrane.

Factors which determine effectiveness of drug action. Interpretation of pharmacological results is plagued by the different amounts of the same substance required for action upon different synapses.[52] In some cases, this is clearly ascribable to the different accessibility of various synapses to the drug. Thus, arterially injected prostigmine does not affect the synapses of Renshaw cells, but it acts upon them when it is introduced into the vicinity of the cells by direct injection (ECCLES *et al.*, 1956). Different potency of synaptic transmission, which is known to occur even at different sites of the same cell (cf. Fig. 14), can also serve to account for different sensitivity. It has been suggested (TOMAN, 1955; GRUNDFEST, 1956b,d) that similarly, and even differently, electrogenic membrane may be composed of molecular structures differing in subtle detail, as the various biologically active steroids etc. differ. This variation may be invoked to account for the

[51] Succinylcholine, on the other hand, blocks the effects of both vagal and sympathetic stimuli on the cat heart (PURPURA and GRUNDFEST, 1956c). This action is not unexpected on the basis of the foregoing analysis. An interesting new example of the correlation between electrogenic and pharmacological effects is provided by the hearts of *Lampetra* and *Myxine* (AUGUSTINSSON *et al.*, 1956). The vagus is an accelerator nerve in the former, and therefore the electrogenesis at the cardiac pacemaker is presumably depolarizing. Acetylcholine and its analogue (butyryl-; propionyl-) also accelerate, while d-tubocurarine and C_6 antagonize this. Eserine also blocks acetylcholine action. The *Myxine* heart apparently has no innervation and does not respond to acetylcholine. The effects on *Lampetra* fully coincide with the actions of the same drugs on muscle (BURNS and PATON, 1951).

[52] This may not be only a peculiarity of the synapses, but also of the pharmacological interest in clinical requirements. A major effort has been to find substances which affect neuromuscular transmission with as little action on other systems as possible. Therefore, we need not be surprised that most synaptic agents available act more on these synapses than elsewhere. Indeed, those compounds which act better on central synapses often develop such drastic effects in the whole organism as to be considered general poisons. Strychnine, which does have some use as a tool for study of synaptic action is a case in point.

frequent finding (e.g. RIKER and WESCOE, 1951) that the potencies of two drugs of similar action diverge in different types of tests. Structural differences of the same kind could also account for differential sensitivity of different tissues (e.g. white and red muscles) or of different species (cf. PATON, 1951). Thus, a number of special conditions, but of a relatively simple type, and some experimentally demonstrable at present, can serve to account for differences in potency of synaptic agents. The data on eel electroplaques show, however, that a number of the more common synaptic drugs (Table 1), some the more active in some systems, others in other systems, all belong to one of two categories, synapse activators or synapse inactivators, although the range of concentrations of the different drugs needed to cause these effects is large.

Re-enforcement and antagonism. The similar actions of different members of the same group appear to explain adequately the re-enforcement which one drug of a given category will exert upon the action of another of this class. Antagonistic actions of members of the opposite groups probably represent a more complex, dynamical situation. The competitive, antagonistic aspect is probably a mass action phenomenon in which two substances[53] react with the same substrate. In the case of synapses, the substrate is an electrogenic membrane, and the dynamics of the combinations manifest themselves as a net balance of electrical activity. The quantitative effects ought to depend upon the relative combining powers (to use the terms of one of the possible mechanisms) of the two substances with their substrate, and should also exhibit a saturation phenomenon if the amounts of drugs used are in excess to the available substrate ("receptor" in the schema). Quantitative comparisons of this type were not done on the eel electroplaque preparations. However, depolarization by the synapse activating substance is prevented in these cells by the inactivator drugs (ALTAMIRANO *et al.*, 1955b). Synaptic excitability is lost but, since depolarization does not develop, the electrically excitable membrane remains responsive to direct stimuli (cf. also CHAGAS and ALBE-FESSARD, 1954) although it develops graded responsiveness.

IX. 3. *Multiple effects*

Eel electroplaques illustrate another aspect of pharmacological action,

[53] Only one of the two participants need be a recognized synaptic agent, and of either category. The other substance may be, for example, heparin, which is an anticurare agent for neuromuscular synapses (cf. CHEYMOL *et al.*, 1955) and for cortical dendrites (PURPURA and GRUNDFEST, 1956b). Likewise, adrenaline is not generally considered as a synaptic agent for cholinoceptive membrane. Nevertheless it antagonizes decamethonium action on neuromuscular transmission strongly, but that of d-tubocurarine very little (DALLEMAGNE and PHILIPPOT, 1952). On the other hand, stimulation of sympathetic nerves can enhance synaptic responsiveness of fatigued muscle (Orbeli effect, HUTTER and LOWENSTEIN, 1955).

the different effects of a drug on different types of electrogenic membrane in the same cell. Not only do the curare-like compounds inactivate synaptic transmission (Fig. 20), they also affect the response of the electrically excitable membrane, but more slowly. The response becomes graded (Figs. 28, 29), and may disappear, at longer times and with higher concentrations. Acetylcholine also exerts an action on the electrically excitable membrane, directly and not through the depolarization of the postsynaptic component (unpublished experiments of ALTAMIRANO *et al.*, 1955b). An electroplaque with its synapses inactivated (e.g. by d-tubocurarine) is not depolarized by acetylcholine. Nevertheless, the electrically excitable, but now only gradedly responsive membrane, gradually becomes inexcitable.

The electroplaque appears to be particularly amenable to these direct actions, whereas most other cells are strongly resistant to d-tubocurarine, acetylcholine, and many other synaptic drugs. Differences based presumably on the accessibility of the electrically excitable membrane to the drug, but probably also on thresholds, are therefore to be expected in various systems. The underlying differences in membrane structure may be revealed by comparative studies of these properties. However, in the context of fundamental theory the explanation for profound overt differences in effect reduces to the trivial —the drug does or does not act because it can or cannot attack.[54]

IX. 4. *Diphasic actions*

Heightened responsiveness of the synapse, whether by increased excitability or by increase in the amounts of transmitter agent available, followed by subsequent depressions, is a characteristic of the action of many drugs. It is illustrated in the increased response of a group of electroplaques to a weak nerve volley (Fig. 23). Both eserine and prostigmine, at first, increase synaptic responsiveness, perhaps both acting as anticholinesterase, which protects the transmitter agent from rapid destruction and thereby increases its effectiveness at synapses of marginal potency. This initial potentiation by anticholinesterases of synaptically evoked responses at other cholinoceptive synapses is well known. The subsequent block produced by both drugs has different causations as described earlier (Figs. 17, 20).

Another type of diphasic action is the secondary depolarization produced by DFP long after its synaptic blocking action (Table 1). DFP does not depolarize axons (TOMAN *et al.*, 1947) at least in short-term experiments. The electroplaque conductile membrane may

[54] The site of attack is an interesting problem. The endplate is sensitive to acetylcholine externally applied, but not to that injected within the structure (DEL CASTILLO and KATZ, 1955a). The squid giant axon can tolerate very high concentrations of acetylcholine injected into the interior, but not very low concentrations (GRUNDFEST, 1953).

behave differently in this respect. However, in prolonged action the drug may have altered the postsynaptic membrane, or have itself been altered by enzymatic processes at this membrane. The available experiments do not permit a decision.

Continued application of synaptic activating drugs also produces diphasic effects (cf. FATT, 1954; ZAIMIS, 1954). In frog muscle (THESLEFF, 1955a,c; but not in rat diaphragm, THESLEFF, 1955b) and in autonomic ganglia (KRIVOY and WILLS, 1956) depolarization subsides in the presence of a large variety of these compounds, including acetylcholine. Transmission still remains blocked in the frog muscle, but not in the autonomic ganglion. These different effects may be ascribed to several phenomena. One might be a slight modification of the postsynaptic membrane which converts the action of certain activating drugs into inactivation. Another possibility is a change in the drug itself, as mentioned above. Of interest for both of these types of change is the close structural relation found between activating and inactivating compounds. Substitution of one ethyl in the end complexes of decamethonium results in a drug which blocks transmission in muscle with only a slight, evanescent initial depolarization (THESLEFF, 1955c). The di- and tri-ethyl homologues do not depolarize and are more powerful blocking agents than is decamethonium.

Another factor might be the decrease of sensitivity of the postsynaptic membrane to the drug (THESLEFF, 1955b; KRIVOY and WILLS, 1956). Thus, after depolarization and block are lifted in autonomic ganglia, addition of more of the activating drug again causes depolarization.[55] Finally, the compound may also exert slower actions on the electrically excitable membrane, as in the eel electroplaque (cf. KRIVOY and WILLS, 1956). Decamethonium appears to raise the critical firing level for the spike of frog muscle about 20 per cent (THESLEFF, 1955a) and seems also to increase markedly the time constant of the membrane (cf. his Fig. 7).

The effects caused by denervation also emphasize the labile nature of the membrane molecular structures. In denervated muscle, d-tubocurarine initiates fibrillatory potentials which originate at the

[55] THESLEFF (1955a) finds that end-plates subjected to prolonged action by acetylcholine or other depolarizing drugs do not react to further additions of the drug, but (personal communication) become depolarizable again after prolonged washing. In addition to the possibility that the sensitivity of the membrane may have been temporarily altered, it is perhaps possible that the type of electrogenesis was temporarily altered, at least in some units. In Section VI, 12, it was pointed out that hyperpolarizing and depolarizing postsynaptic membranes fundamentally appear to be related (cf. also GRUNDFEST, 1956b,d). Conversion of one electrogenic type to the other is not inconceivable. It would, for example, require only the inactivation of sodium "valves" to change the depolarizing postsynaptic membrane into the hyperpolarizing variety. Temporary changes of this kind in part of the electrogenic population could account for repolarization of the muscle end-plate and its subsequent insensitivity to added acetylcholine.

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endplates (JARCHO *et al.*, 1951). This demonstration of a specific change in the postsynaptic membrane is presumably part and parcel of the profound effects caused by denervation not only in muscle but in many other organs as well, including those seen as transneuronal degeneration in the central nervous system. Since so little is known as yet about the molecular structures of excitable membranes, except the fact that they are labile, it seems unprofitable to speculate further on the nature of the changes disclosed by these various diphasic actions. Nevertheless, as the foregoing attempted to indicate, it appears unnecessary, at least for the present, to abandon explanation of the synaptic actions of drugs in terms of their known effects on the electrogenic mechanisms.

IX. 5. *Generalized effects*

Two rather different types of effect will be treated under this heading. Both are more apt to occur in the intact organism than in simpler systems, and not at all in the simplest, isolated preparations such as nerve-muscle, or nerve-electroplaques.

(*a*) Depolarization, even by a small amount, may be expected to upset the electrochemical steady state of muscle fibres which form a large portion of the body mass. This may cause significant ionic shifts. The resulting changes in the interstitial or even in the circulatory fluids might therefore cause generalized effects on the muscles themselves and on other organs. Were the change to affect hormonal balance, the central nervous system or other particularly strategic (e.g. pulmonary) tissues, the results in the organism might be long-lasting and drastic. ZAIMIS (1954) has reported potassium shifts following administration of synapse-activating drugs.

(*b*) As noted above, the primary interest of pharmacologists is concentrated on the agents affecting transmission in muscle. However, the effects on other synapses may lead to more generalized results. While these are often exerted clearly only with higher concentration of the drugs, some minimal action may also be expected. These effects on highly integrated mechanisms may become disturbing to the organism. The side effects which the drugs produce in this way need not be initiated through the central nervous system. For example, mechano-transducer membrane in various sensory receptors is also excited by chemicals (e.g. SKOUBY, 1953; GRANIT *et al.*, 1953; WIERSMA *et al.*, 1953), which may be considered an inadequate stimulus for the cells (cf. GRUNDFEST, 1956b,d). Generalized side effects on receptor membrane might then react upon the central nervous system.

Summary. Pharmacological actions of drugs may be viewed firstly as manifestations either of inactivation of electrogenic responses in postsynaptic membrane or as an activation of these. The latter effect

leads to electrogenesis of a type determined only by the nature of the postsynaptic membrane. This electrogenesis may in turn affect the electrically excitable membrane. The actions may be excitatory, inhibitory, or a combination of the two. The drugs of both categories also exert actions directly on the electrically excitable membrane, but these probably develop more slowly than do the effects on the synaptic systems. All these electrogenic actions therefore need to be considered separately and in combination in the analysis of pharmacological effects.

X. Evolution of Electric Organs

Discussion of this general problem has been postponed until now, because the study of the mechanisms of single cells throws the matter in a perspective different from that formulated by early workers (Du Bois-Reymond, 1881; Fritsch, 1883). The latter were concerned with and sought to answer two questions: (i) what did the organs evolve from? and (ii) what were their evolutionary steps? The first question was settled by the discovery, as Redi had guessed earlier, that the electric organs originated from muscle in all but *Malapterurus*, and that in the latter the electroplaques are probably modified secretory cells. The second problem, concerning the evolutionary process, was more difficult to resolve, but seemed to be settled by the discovery of fishes with small electric activity or possessed of structures which had the appearance of electric organs, but had a "pseudo-electric" function (Du Bois-Reymond, 1881, p. 68). As mentioned earlier, Sachs had discovered such structures in *Eigenmannia* (*Sternopygus*) *virescens*, but Fritsch (Du Bois-Reymond, 1881) claimed that neither these nor the cells of *Sternopygus carapo* (probably the present *G. carapo*) were true electric organs. Thus there appeared to be an evolutionary hierarchy to satisfy the question.

However, at least in a number of *Mormyrids* and *Gymnotids* (cf. Lissmann, 1951; Coates *et al.*, 1954; Couceiro *et al.*, 1955b) including both *E. virescens* and *G. carapo*, electric discharges have now been found—small it is true, but this fact is accounted for by the small number of series cells. Therefore, the discharges of the individual cells can have nothing "rudimentary" about them. Indeed, the remarkable rhythmicity of their discharge and the fact that the activity persists continuously throughout the observed life of the fish indicates (i) that the nervous control may be of an extremely high order of development and (ii) that the processes for replenishing the transmitters are much better developed in these than in the larger forms. These species might therefore with equal reason be considered as representing a higher evolutionary stage rather than a lower.

The low voltage and prolonged discharge of *Raia* similarly cannot

be a more primitive type of response. The size of the discharge is small for two reasons: (i) there are fewer series elements and (ii) the discharge of each is without overshoot and is a simple depolarization. However, this appears to be a response of a vestigial endplate, in which the electrically excitable membrane had disappeared. Therefore the *Raia* electroplaque is no more primitive than is the endplate of a muscle fibre.

In the *Torpedines* the response is also probably of an endplate, but perhaps of one similar in properties to the neurally excitable membrane of the eel electroplaque except that it is more densely innervated and therefore responds more uniformly and with a larger amplitude than does the p.s.p. of *Electrophorus*. Whatever is the nature of the organ in *Malapterurus* it can hardly be classified on an evolutionary scale because it is apparently aberrant in origin.

It is therefore unlikely that any of the extant and presently known species of electric fishes represent a primitive evolutionary stage, and it is highly improbable also that palaeontology will fill that gap. However, electric organs appear widely in two groups, the elasmobranch *Raiadae* and *Torpedinae* and the teleost *Gymnotidae* and *Mormyridae*, as well as in a few strays (*Astroscopus*, *Malapterurus*, etc.). Mutation, unknown to the older workers, is the most reasonable explanation. In the *Gymnotidae*, this mutation took the form of eliminating the contractile elements of muscles, leaving behind both the endplate and the electrically excitable membrane. In the elasmobranchs only endplates were left. The electric organs therefore appear to represent several stages in a new, mutational development which is based upon the older electrogenic structure of muscle (and secretory cells in *Malapterurus*). The electroplaques of *Electrophorus* and of other spike generators indicate one stage, those of the *Torpedines* at least one more, and possibly two if the endplate types of responses of some also have a graded sodium-conductance type of overshoot. These developments are associated with a related modification, that of unresponsive membrane at the noninnervated surfaces.[56]

XI. Conclusion and Summary

The electric organs, in all but *Malapterurus*, are structures derived from muscle, capable of discharge by the innervating control of the

[56] This last remarkable modification should repay intensive study. The noninnervated surfaces might, for example, be identical with the postsynaptic membrane, but not responsive because they are not innervated nor electrically excitable. Decisive evidence on this could be obtained by applying jets of chemical excitant (Nastuk, 1953b; del Castillo and Katz, 1955a), particularly with the *Torpedine* cells, which respond electrogenically to acetylcholine (Feldberg and Fessard, 1942). In view of present-day concepts of experimental embryology, this possibility is unlikely, because it would create a difficulty for accounting for the remarkably regular innervation of only one part of the electroplaque surface.

central nervous system. One general feature of all is that they have undergone a special modification which permits series addition of the voltages generated by the individual electroplaques of which the organs are composed. This modification consists in a differentiation of the cell membrane, only that limited surface which is innervated being capable of an electrogenic response. As the membrane is modified muscle it is not unexpected that only one surface of it is innervated, nor that probably all the innervated faces in a row of electroplaques are symmetrically aligned. The electric organs offer a particularly clear demonstration of differently acting electrogenic mechanisms. Certainly in *Raia*, and very probably also in the torpedines, the electroplaque is a homologue of the muscle endplate, the conductile electrogenic membrane and the mechanically responsive elements of the muscle fibre being lost. These electroplaques, like the endplate, do not respond to electrical stimulation, but are synaptically excitable by their nerves. In *Torpedo*, as in muscle, the postsynaptic membrane is demonstrably cholinoceptive, injection of acetylcholine into the organ producing electrogenesis in the latter. These electrical responses are depolarizing, as is that of the muscle endplate, although a special modification of the electrogenic mechanism may be expected in *Torpedo*. Another special mechanism may be expected in the electroplaques of the *Malapterurus* organ, which is probably derived from secretory cells, but this modification is one which can be accounted for on the basis of our present knowledge of electrogenic tissues.

In *Electrophorus*, and perhaps also in the other teleost electric fishes, the electroplaque retained the conductile electrogenic membrane of the muscle fibre, as well as the endplate type of membrane, but dispensed with the contractile machinery. These electroplaques therefore possess two electrogenic mechanisms. One is neurally evoked, as is the endplate potential; the other is electrically excitable and identical in properties with the spike of muscle fibres or of axons.

The electroplaques of the elasmobranchs long ago provided evidence which could have established firmly a distinction between electrogenic membrane responsive only to neural (but not to electrical) stimuli, and therefore neurohumorally activated, and membrane responsive to electrical stimuli. This distinction was made by GARTEN (1910; cf. FESSARD, 1952), but was not incorporated into the body of electrophysiological doctrine. The demonstration of this dual mechanism in the same cell (muscle fibre, DEL CASTILLO and KATZ, 1954b; innervated eel electroplaque, ALTAMIRANO *et al.*, 1955a,b) with intracellular recording has now provided conclusive evidence.

The presence of two different membrane components, endowed with different electrogenic properties and subserving different functions,

accounts for various phenomena in the response of eel electroplaques. The synaptically activated component develops the graded electrical response of the postsynaptic potential (p.s.p.). The electrically excitable component under normal conditions evolves an all-or-nothing spike with temporarily reversed membrane potential, the overshoot. The spike is propagated without decrement along the cell by local circuit stimulation of the electrically excitable elements of the membrane. Stimulation of the cell by its nerve supply first initiates the p.s.p., and this depolarization, in turn, can elicit the spike of the electrically excitable membrane.

The synaptically activated membrane probably develops its electrogenic response, as does the muscle endplate, by increased permeability to all ions. The response is, however, relatively independent of the membrane potential, since it arises in a system which is not electrically excitable. The spike-generating membrane, on the other hand, probably reacts by means of the sequential, potential determined processes of sodium and potassium conductance and sodium inactivation (HODGKIN and HUXLEY, 1952).

The generalization made on the basis of studies of the eel electroplaque and other evidence that the synaptically activated membrane of all post-junctional cells is not electrically excitable (ALTAMIRANO and GRUNDFEST, 1954; ALTAMIRANO *et al.*, 1955a) has been useful in predicting experiments (KAO and GRUNDFEST, 1956, and to be published) and interpreting extant data (GRUNDFEST, 1956a,b,c,d) and has led to the finding that the richly synaptic apical dendritic portions of cortical cells are not electrically excitable (GRUNDFEST and PURPURA, 1956). In the past few years a considerable body of confirmatory data has become available from various invertebrate and vertebrate synaptic complexes, which support this classification of differently excitable electrogenic mechanisms.

The same generalization disposes of the perennial argument between the proponents of electrical and humoral synaptic transmission. The synaptic membrane not being electrically excitable, transsynaptic activation must occur through a chemical intermediate. Thus, in so far as the generalization is found to apply, the problem of synaptic transmission is sharpened to focus on the membrane molecular mechanisms which are involved.

The different molecular constitutions of the synaptically and electrically excitable membrane components, which are reflected in their different electrogenic properties, probably also account for the greater susceptibility of the former to attack by various chemical agents. A comprehensive study of the effects of a number of these drugs on the eel electroplaque has revealed that, as in other synaptic systems, two distinct classes of substances exert different electrogenic effects.

Members of one group inactivate the electrogenic capacity of the postsynaptic membrane, thereby blocking transmission of excitation from the nerve to the electroplaque. However, the electrically excitable membrane remains responsive to electrical stimuli. Members of the second group cause postsynaptic electrogenesis, and the resultant depolarization inactivates the electrically excitable membrane. Stimulation of this, whether by direct stimuli or neural, becomes ineffective, while the postsynaptic membrane, excited through its nerve, is still capable of developing a p.s.p. Transmission of excitation is therefore also blocked by these drugs, but through a different instrumentality. The analysis of these different phenomena, in terms of the electrogenic bases, which has been carried out with eel electroplaques, is also applicable to other synaptic systems and thereby helps to clarify pharmacological manifestations as effects upon different electrogenic components of cells.

The synaptic drugs also exert effects upon the electrically excitable membrane of the eel electroplaque. Of particular interest is the transformation, by the synapse inactivating drugs, of the explosively responding electrically excitable membrane to one developing graded electrical activity. Then, however, the maximum of response has the same peak e.m.f. as that generated normally during the spike. Graded responsiveness of the electrically excitable membrane also occurs during early relative refractoriness of the eel electroplaque, squid, earthworm, and crayfish giant axons and cardiac muscle, and can be induced by drugs acting on the squid giant axon. It appears likely that some invertebrate muscles respond normally only with graded responses of electrically excitable membrane.

The eel electroplaque, in which the synaptically activated membrane component is diffusely disseminated amongst electrically excitable membrane, resembles the neuron of the spinal cord more closely in this respect than it does the muscle fibre. However, it does not possess the hyperpolarizing p.s.p. of inhibitory function which is found in cells of the latter variety. Nevertheless, as the foregoing has indicated, study of the properties of the electroplaque has helped to elucidate the problems of synaptic transmission in the neuron: firstly, as involving differently excitable electrogenic mechanisms; second, that the synaptically excitable component and the electrically excitable have different pharmacologic susceptibilities which are manifested through electrogenic mechanisms specifically different in properties.

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R. Wurmser and S. Filitti-Wurmser

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THERMODYNAMIC STUDY OF THE ISOHAEMAGGLUTININS

R. Wurmser and S. Filitti-Wurmser

THE main interest of studies of the thermodynamic properties of antigen-antibody reactions is to supply data which may be used for the calculation of the energies of formation of the complexes. The direct determination, by calorimetry, of the energy of the linkage between antigen and antibody has been made only in the case of the haemocyanin anti-haemocyanin complex (BOYD *et al.*, 1941). All other estimations were based on equilibrium measurements.

The information provided by such measurements permits an attack on many problems. The calculation of the energy of association may be attempted on the basis of the structure of the specific group of the antigen. This energy may then be compared with the experimental value.

The measurements also give information concerning the number of the specific groups per molecule. Finally, as the results obtained with the isohaemagglutinins show, the thermodynamic study may supply also information on the carriers of the specific groups, which is of obvious interest from the point of view of the biogenesis of proteins.

Since the equilibrium constant is affected by all changes involved in the formation of the complex, the most significant results concerning the specific groups have been obtained by studying the influence of slight modifications of structure of a monovalent hapten on the affinity of this hapten for the corresponding antibody. It may be assumed that in this type of experiment the differences of affinity are exclusively due to the local changes. Thus one may, as PAULING and co-workers (PRESSMAN *et al.*, 1946) did, compare these differences of affinity with the calculated differences due to the various types of forces acting at the site of reaction.

EISEN and KARUSH (1949) and LERMAN (1949) also studied reactions between antibody and monovalent hapten. In their experiments the concentration of free hapten in a mixture with the antibody was directly measured by a dialysis method. Thus the absolute values of the free energy of combination of one hapten molecule were obtained, but thus far no systematic determinations aimed at analysing the structure have been made.

The study of the equilibrium between antibody and antigen is much more complex than that of the equilibrium between antibody and mono-

valent hapten since in agreement with prevailing opinion—both antibody and antigen contain multiple specific combining sites. Consequently there are complexes of different composition. In order to measure an equilibrium constant in such a system it is necessary to know both the concentration of free antibody and that of two defined complexes.

One attempt at this type of investigation was made by HAUROWITZ, CRAMPTON and SOWINSKI (1951). In their work, the apparent solubility of azoprotein-rabbit antibody precipitate is considered as the concentration of free antibody A produced by the dissociation of the precipitate GA_n, according the assumed equation, where G represents the antigen

$$GA_n \rightleftharpoons GA_{n-1} + A$$

A more promising method is that of GOLDBERG (1952) used by SINGER and CAMPBELL (1953). This method applies to the antigen-antibody system the statistical theory of FLORY-STOCKMAYER of the molecular-size distribution of branched chain polymers. A basic assumption is that of the absence of cyclical structures in the antigen-antibody complex. Furthermore it is assumed that any unreacted site of the antigen or the antibody is as reactive as any other site regardless of the size or shape of the complex in which it is found. On the basis of this theory it is possible to calculate, from the concentration of free antigen, the concentration of the complexes GA and G_2A and the equilibrium constant of the equation $GA + G \rightleftharpoons G_2A$. However, in the experiments of SINGER and CAMPBELL a value for the equilibrium constant was obtained which decreases as the antigen excess is increased. It must be recognized that, up to the present time, the energetic data available for antigen-antibody reactions remain rather limited.

Since the polyfunctionality of both reactants is the main complication encountered in the thermodynamic study of precipitin reactions, one may expect to find a simpler situation in the case of agglutinin reactions. Regardless of the valence of the agglutinins, it appears unlikely, for geometrical reasons, that the number of molecules simultaneously bound to two cells would be considerable as compared with the total number of molecules bound by a single valence. Moreover the experimental technique, in the work on the equilibrium between agglutinins and agglutinogen groups, is facilitated by the possibility of separating the total amount of combined antibodies by centrifugation. There still remains the complication caused by the heterogeneity of antigens and antibodies. This is, no doubt, responsible for the results obtained in the fixation of agglutinins by the typhoid bacillus (EISENBERG and VOLK, 1902). In this case, as in the absorption of haemolysin by the red cells (CROMWELL, 1923), the variation of fixed antibody as a function of its concentration can be represented by FREUNDLICH's isotherm. This

relation is obtained, as indicated by HINSHELWOOD (1940), when the surface possesses regions with varying reactive capacities.

The isohaemagglutinins present a much higher degree of homogeneity which greatly facilitates their study. However, no systematic investigation of these substances was attempted until some ten years ago. The present review is almost exclusively concerned with the experiments carried out in our laboratory. Of the numerous questions that were raised by these experiments, only a few have been thus far further investigated. As noted above, the essential part of the work carried out with the aid of thermodynamic methods on the antigen-antibody reaction is concerned with the structure of the specific groups. In our study of human isohaemagglutination we have been led to look at the problem from a quite different angle. Facts which emerged from our earlier observations focused our attention on molecular properties of the isoagglutinin other than the configuration of the specific sites.

I. THE ISOHAEMAGGLUTINATION

Isohaemagglutination discovered by LANDSTEINER is the agglutination which is produced when one mixes human red cells with sera belonging to the human species. Human beings can be classified, from this point of view, into four blood groups: O, A, B, AB and six principal subgroups A_1, A_2, B, A_1B, A_2B and O, according to the type of antigens on the red cells. An agglutinin active against one of these antigens is found in a person's serum only if his own red cells do not contain this antigen. Finally, the blood group to which an individual belongs is an inherited character, controlled by a series of at least four alleles.

II. REVERSIBILITY OF ISOHAEMAGGLUTINATION

The first point to establish for a thermodynamic study of isohaemagglutination is the reversibility of the process. Evidence for this reversibility has been provided by the experiments of FILITTI-WURMSER and JACQUOT-ARMAND (1947). They displaced the equilibrium of a mixture of group B red cells and group A serum by varying the temperature and by dilution. The method consisted of measuring the yield of agglutination, that is to say, the ratio between the number N_a of agglutinated red cells in 1 cu. mm and the number N_t of red cells present in the same volume. The number N_a was obtained by the difference between N_t and the number of red cells remaining free after a time interval of shaking necessary to attain the equilibrium. All counts were made with haemacytometers directly, or on microphotographs.

1. In a series of experiments it was shown that the same yield of agglutination is obtained when the reaction is carried out at a relatively high temperature 37°C or when the reaction is carried out in two steps, the first at a low temperature (4°C) and the second at 37°C.

2. Instead of dissociating the agglutinates by raising them to a higher temperature, they may be dissociated by dilution. One thus finds that the same yield of agglutination is attained if a mixture of red cells and serum is made directly in a definite volume V, or if the operation is carried out in two steps; mixing in a small volume and then adding buffer to the volume V.

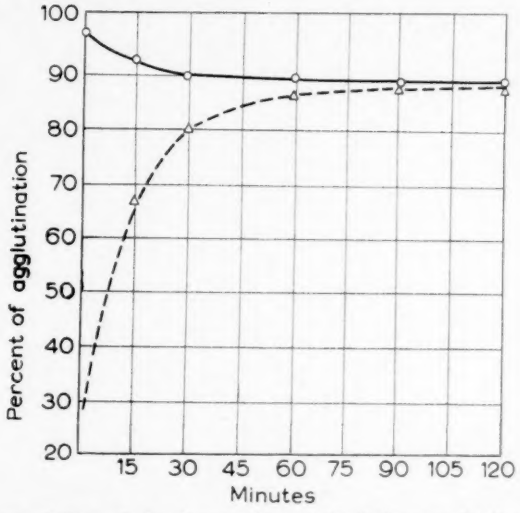

Fig. 1. Reversibility of the human B- anti-B isohaemagglutination. The per cent of agglutination is the same from whichever side it is approached. (Taken from WILKIE and BECKER, 1955.)

The reversibility of the isohaemagglutination of A red cells by sera of group B has been demonstrated in the same manner by MAVRIDES (1954).

An excellent experiment of WILKIE and BECKER (1955a) confirms this reversibility. A mixture of serum and B red-cell suspension was placed on a shaker. At appropriate intervals of time, aliquots were withdrawn and the number of free cells per cubic millimetre determined. The lower curve of Fig. 1 represents the results. Duplicates of the same mixture were centrifuged for one minute at 65× gravity, the cell pack resuspended, and placed on a shaker; at appropriate intervals, the free-cell count was determined. The upper curve of Fig. 1 gives the results. It is apparent that the yield of agglutination was independent of the direction from which it was approached.

III. Classification of Anti-B Sera

Owing to the red-cell counting technique it was found that the anti-B sera could be separated into classes in which each serum has the same quantitative effect (FILITTI-WURMSER, JACQUOT-ARMAND and WURMSER, 1950). This fact appeared while studying the influence of the

temperature on the maximum number of agglutinated red cells per cubic millimetre by a serum. Fig. 2 shows the results obtained in the determination of the maximum number N_θ of B cells agglutinated by a given serum at two temperatures. In order to represent all measurements in one figure, the logarithm of the number N_t of red cells present

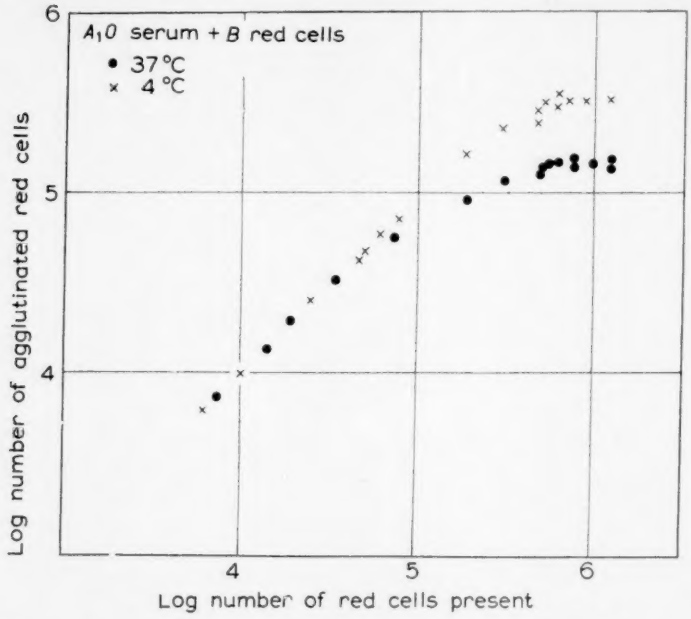

Fig. 2. Agglutination in a mixture of serum with a varying number of red cells, at two temperatures.

instead of the number itself has been plotted against the logarithm of the number N_a of agglutinated red cells. At each temperature N_a increases as a function of N_t and attains a maximum value N_θ. It is apparent that the maximum number of red cells agglutinated increases as the temperature decreases.

For comparative purposes the ratio of the maximum number N_4 at 4°C to the maximum number N_{37} at 37°C has been determined for numerous sera. First it was found that the heating of sera at 56°C for 50 minutes produces changes of N_4/N_{37}. This treatment has the effect of destroying an inhibitor whose action varies with temperature, and which has been identified with the complement (ZERMATI *et al.*, 1947; AUBEL-LESURE, 1950).* To account for the presence of this inhibitor, all the measurements which will be described below and in other sections were made with heated sera.

* However, according to a recent communication of M. H. WILKIE (1956 Federation Proc. 15, 622) this identification is not established.

In addition, the absence of nonspecific "cold" agglutinins was always controlled. If they existed at all, they were absorbed by shaking the sera with washed O cells at 4°C.

In Table 1 the values of N_4/N_{37} found for 59 sera are reported. There appear to be four distinct classes into which the data may be separated. The numerical proportion of the different classes suggests a genetic correlation. If one considers the 41 sera of subgroup A_1 taken at random, one sees that 80 per cent of them have the same value $2{\cdot}5 \pm 0{\cdot}2$ for the N_4/N_{37} ratio. According to THOMSEN's data, 78 per cent of the individuals in the A_1 subgroup have a genotype A_1O. This proportion is very close to the proportion of the sera with an N_4/N_{37} ratio equal to 2·5. As a matter of fact a number of these sera were identified to be of the A_1O genotype. The donors had an A_1B father and a B mother, or a B father and an A_1 mother.

On the other hand it was expected that the A_1 sera of which the N_4/N_{37} ratio are close to 1·3 would be of the homozygote genotype A_1A_1 or heterozygote genotype A_1A_2. In agreement with this postulation the N_4/N_{37} ratio of 1·3 was found both for sera unquestionably of the A_1A_1 genotype and for those which were probably of the A_1A_1 genotype (FILITTI-WURMSER *et al.*, 1954). The two other classes of sera listed in Table 1 belonging either to subgroup A_2, ($N_4/N_{37} = 2$), or to group O, ($N_4/N_{37} = 1{\cdot}2$), obviously do not present any difficulties of characterization.

The simplest assumption that could be made concerning this possibility of classifying the anti-B sera is that the sera of a given genotype contain a unique isohaemagglutinin characterized by the effect of temperature on its action with B red cells. The anti-B isoagglutinins will be designed by the symbols: $\beta(OO)$; $\beta(A_1A_1)$; $\beta(A_1O)$; $\beta(A_2O)$, etc.

In favour of this view there is the argument that in the sera with the higher N_4/N_{37} values, it has been demonstrated (FILITTI-WURMSER and JACQUOT-ARMAND, 1947), that the fraction of agglutinin not absorbed at 37°C agglutinates more red cells at 4°C than at 25°C and more at 25°C than at 37°C. Consequently one is not dealing here with a mixture of "hot" and "cold" specific agglutinins. The temperature effect is due to the exothermicity of one and the same reaction. None of the measurements which are going to be described contradict this hypothesis of homogeneity. But the degree of this homogeneity, to what extent it is valid, will be discussed in Section VII.

IV. THERMODYNAMIC STUDY OF THE ISOHAEMAGGLUTININS

1. *The primary stage of agglutination*

Up to now we have been dealing with the total phenomenon of agglutination, that is to say the combination of the isoagglutinin with the agglutinogen group of the red cells as well as the subsequent process of

aggregation. It is the second stage, much less rapid than the first one, which necessitates the long agitation in order to reach the equilibrium state of agglutination. The mechanism of aggregation is obviously very complicated. Whatever the nature—specific or not specific—of the

TABLE 1

Maximum Number per cubic millimetre of B *Red Cells Agglutinated* $\times 10^{-3}$ *in a mixture of* 0·6 *ml heated serum* + 0·3 *ml Suspension of* B *Red Cells in Buffer;* N_{37} *is at* 37°*C;* N_4 *is at* 4°*C*

Sera	N_{37}	N_4	N_4/N_{37}	*Sera*	N_{37}	N_4	N_4/N_{37}
			A_1 *Sub-Group* A_1O				
1	270	742	2·74	17	1,200	2,700	2·25
2	375	1,015	2·70	18	982	2,720	2·76
3	346	980	2·83	19	1,700	3,922	2·30
4	393	1,090	2·77	20	3,000	7,540	2·51
5	400	1,000	2·50	30	810	2,000	2·46
6	520	1,200	2·30	31	950	2,360	2·48
7	520	1,254	2·41	32	1,000	2,200	2·20
8	760	2,100	2·76	33	400	950	2·37
9	800	2,030	2·53	34	3,500	7,800	2·22
10	700	1,680	2·40	35	2,800	6,200	2·21
11	800	1,900	2·37	36	1,100	3,000	2·72
12	1,000	2,500	2·50	37	1,300	3,100	2·38
13	1,000	2,500	2·50	38	800	1,900	2·37
14	930	2,422	2·60	39	900	2,360	2·62
15	1,000	2,450	2·45	40	1,900	4,100	2·16
				56	2,560	6,600	2·53
				57	1,600	3,700	2·31
			A_1A_1				
21	1,160	1,680	1·44	25	1,500	1,772	1·18
22	5,000	6,900	1·38	41	1,800	2,000	1·11
23	5,500	7,550	1·37	42	2,100	2,900	1·38
24	5,500	6,600	1·20	43	13,600	19,000	1·39
			A_2 *Sub-Group*				
26	1,000	2,000	2·00	44	1,300	2,600	2·00
27	800	1,600	2·00	45	1,800	3,300	1·83
28	890	1,850	2·07				
			O *Group*				
46	2,500	3,300	1·32	51	5,000	6,200	1·24
47	3,000	3,600	1·20	52	6,700	8,000	1·19
48	2,700	3,400	1·25	53	6,500	7,500	1·15
49	6,700	8,000	1·19	54	11,000	14,000	1·27
50	2,900	3,300	1·13	55	3,700	4,100	1·10
				58	1,100	1,460	1·32
				59	2,000	2,600	1·30

cohesive forces which exist between the agglutinated cells, there are great difficulties in devising a quantitative theory for such a process. By contrast, the theoretical treatment of the primary stage is simplified by a fact already indicated in the introduction. Considering the spatial arrangement of the aggregated red cells, it is evident that, for geometrical reasons, even if the agglutinin is "multivalent," a very small number of its molecules would have more than one specific site combined with the agglutinogen group. Thus one may expect that the agglutinin, whether it is or is not "monovalent," will behave as a monovalent reactant. If then one considers a red cell as a large molecule, one ought to be able to apply the mass-action law, following the theory of multiple equilibria (FILITTI-WURMSER *et al.*, 1950). Only a small proportion of agglutinogen groups on a red cell will be combined. These will react as if they existed as independent molecules. If moreover the agglutinogen groups are identical, an apparent intrinsic constant K may be calculated which characterizes the association of an isoagglutinin molecule with an agglutinogen group.

The theory of multiple combinations has been established and the argument discussed by LINDERSTRÖM-LANG (1924), VON MURALT (1930), WYMAN (1943), and KLOTZ (1946). The association constant corresponds to the equilibrium between red cells carrying n molecules of isoagglutinin fixed on n sites specified by their position and red cells carrying, in addition to the above, an extra molecule of agglutinin on another similarly specified site. The ratio r_c of the number of moles of isoagglutinin combined to the number of moles of red cell present is

$$r_c = \frac{mK(A)}{1 + K(A)} \qquad \ldots.(1)$$

where m is the number of agglutinogen groups per red cell, and (A) the concentration of free agglutinin in the equilibrium state.

2. *Estimation of the isoagglutinin concentration in relative value*

In order to apply relation (1) to the study of isohaemagglutinins it is necessary to have a method for dosage of these substances which is valid for small concentrations of the order of 10^{-10} moles per litre. The old technique "two-fold dilution" which consists of noting the highest dilution corresponding to the lower threshold of agglutination is not precise enough, since the threshold is not defined sufficiently. The method of HEIDELBERGER and KENDALL (1929), founded on microdeterminations of nitrogen, is, in spite of its high sensitivity, still below that which is necessary to study systems where the concentration of the isoagglutinins varies between 10^{-8} to 10^{-10} moles per litre. The method which has served for this thermodynamic study permits one to obtain the concentration of the isoagglutinins in relative values with

an accuracy of 5 per cent (FILITTI-WURMSER *et al.*, 1950). It consists of measuring under reproducible conditions the power of agglutination, expressed as the maximum number of agglutinated red cells. In order to know the concentration in absolute value, it is necessary to establish a coefficient describing the relation between the agglutination power and the molarity of the agglutinin. This determination was done for each type of isoagglutinin in a zone of concentrations sufficiently high to permit good micro-nitrogen determinations. Since the agglutination is an exothermic process, there is an advantage in choosing, for the relative measure of the isoagglutinin concentration, the maximum number of agglutinated red cells at a temperature as low as possible, but sufficiently distant from the freezing point. For experimental conditions the temperature of 4°C and a mixture of 0·6 ml of serum with 0·3 ml of a suspension of red cells in a buffer were chosen. The shaking of the mixture must be continued until the equilibrium is reached (60 minutes at 4°C). The maximum number of agglutinated red cells N_4 is proportional to the concentration of the isoagglutinin, as is shown in Table 2.

TABLE 2

Proportionality of the Maximum Number N_4 to the Concentration of Serum in the Mixture of Serum Plus Red Cell Suspension

Anti-B *serum**			*Anti*-A *serum*†		
Dilution	$N_4 \times 10^{-3}$	$N_4 \times$ *dilution* $\times 10^{-3}$	*Dilution*	$N_4 \times 10^{-3}$	$N_4 \times$ *dilution* $\times 10^{-3}$
1:10	740	7400	1:3	873	2619
1:15	554	8310	1:4	633	2532
1:15	533	7995	1:6	417	2502
1:15	566	8490	1:10	253	2530
1:15	495	7425	1:20	133	2660
1:20	380	7600			
1:30	264	7920			
Mean value: 7880 ± 400			Mean value: 2570 ± 60		

* FILITTI-WURMSER *et al.* (1952).
† MAVRIDES (1954).

3. *Application of the mass-action law*

Relation (1) may be arranged in a linear form such that

$$\frac{1}{r_e} = \frac{1}{m} + \frac{1}{mK(A)} \qquad \ldots.(2)$$

We shall be using relation (2) except in the discussion of the homogeneity of the agglutinins (Section VII) where another mode of presentation of results is more convenient.

Let us consider a mixture at equilibrium containing N_t red cells per cubic millimetre in the presence of a more or less diluted serum at a given temperature. The relative agglutinin concentration of the serum was, before the addition of red cells, measured by the number N_4. One centrifuges and then one looks for the maximum number N_4'

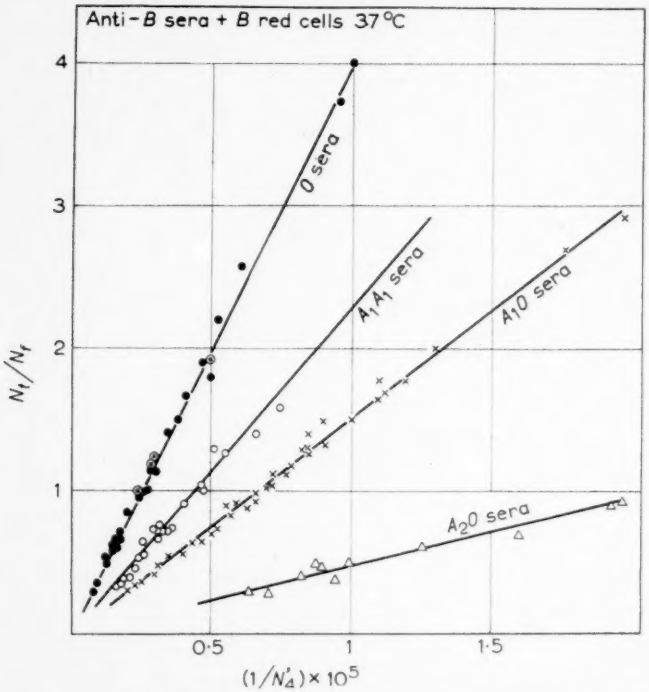

Fig. 3. Binding of anti-B isohaemagglutinins by B red cells at 37°C. Abscissa: reciprocal of the free agglutinin concentration (in relative value). Ordinate: reciprocal of the amount of agglutinin fixed (in relative value) per red cell (equation 3).

of agglutinated red cells in the supernatant. N_4' is proportional to the molar concentration (A) of free agglutinin. The difference $(N_4 - N_4')$ or N_f is proportional to the number (GA) of agglutinogen groups G combined with the agglutinin A in one cubic millimetre. Moreover, N_t is proportional to the total number of group G, combined or not, present in this same volume. The value of r_c is $6 \times 10^{17} N_f/(\phi N_t)$ where ϕ is the ratio N_4/molarity. As the concentration (A) of the agglutinin remaining free is equal to N_4'/ϕ, relation (2) may be written:

$$\frac{N_t}{N_f} = \frac{6 \times 10^{17}}{m\phi} + \frac{6 \times 10^{17}}{mKN_4'} \qquad \ldots . (3)$$

If one mixes different proportions of red cells to serum, one has,

once the equilibrium is attained, different values of N_t/N_f and of $1/N_4'$. Accordingly, if N_t/N_f is plotted against $1/N_4'$, one ought to obtain a straight line whose slope P is $6 \times 10^{17}/mK$.

Fig. 3 represents the results obtained at 37°C with different sera of A_1O individuals, different sera of A_1A_1 individuals, different sera of A_2 individuals, and different sera of group O individuals (FILITTI-WURMSER *et al.*, 1950, 1952, 1953c).

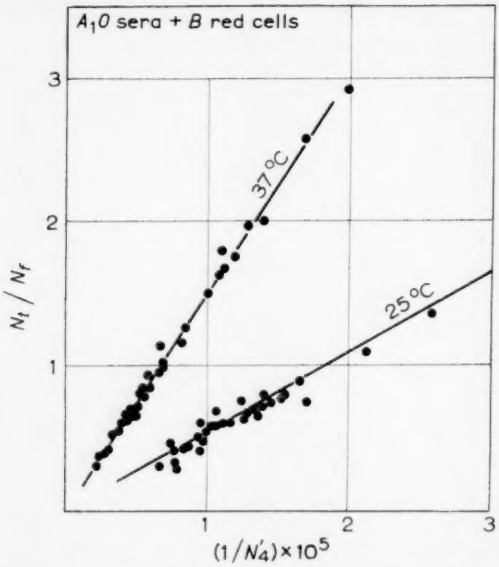

Fig. 4. Binding of the $\beta(A_1O)$ isohaemagglutinin by B red cells at 25°C and 37°C. Same abscissa and ordinate as in Fig. 3 (Equation 3).

It could be argued that the particular characteristics of the anti-B of group O sera was due to the presence of anti-A isoagglutinins. However it has been demonstrated (FILITTI-WURMSER *et al.*, 1952) that the slope P has the same value for the sera deprived of anti-A by absorption on A_1 red cells at 4°C (Fig. 3, encircled points) as that for the nontreated sera.

4. *Determination of the heat of reaction*

Fig. 4 gives an example of straight lines obtained at two temperatures 25°C and 37°C with different A_1O sera mixed with B red cells. The slopes of these lines are respectively $0{\cdot}5 \times 10^5$ and $1{\cdot}5 \times 10^5$. From eq. (3) it follows that the determination of slopes P_1 and P_2 of the lines obtained for the same serum at two temperatures T_1 and T_2 makes possible the estimation of the enthalpy change ΔH:

$$-\Delta H = 4{\cdot}575 \log \frac{P_2}{P_1} \frac{T_1 T_2}{T_2 - T_1} \qquad \ldots.(4)$$

The condition imposed is that the number m of agglutinogen groups on a red cell does not vary with the temperature. There is ample evidence that such a condition is adequately fulfilled.

In the first place MAVRIDES (1950) found the same value of ΔH ($-$ 16 kcal) for $\beta(A_1O)$ by substituting a suspension of stroma for B red cells. This eliminates an influence of a temperature effect on the cellular structure or size. Furthermore unlike the strong response of A_1O sera to the variation of temperature, the response of group O sera is very weak in the presence of the same B red cells.

Thus relation (4) can be applied and one is able to obtain by simple red-cell counts the heat of combination of the various isoagglutinins with an agglutinogen group. Table 3 shows the results which have been obtained until now for anti-B agglutinins (FILITTI-WURMSER *et al.*, 1950, 1952, 1953c) and anti-A (MAVRIDES, 1955).

TABLE 3

Heat of Combination of Isohaemagglutinins by Agglutinogens

Isoagglutinin	*Agglutinogen*	$-\Delta H$ kcal/mole
$\beta(A_1O)$	B	16 ± 2
$\beta(A_1A_1)$	B	$6{\cdot}5 \pm 1{\cdot}1$
$\beta(A_2O)$	B	9
$\beta(OO)$	B	$1{\cdot}7 \pm 0{\cdot}4$
$\alpha(BO)$	A_1	19 ± 3
$\alpha_1(BO)$	A_1	$33 \pm 2{\cdot}5$

V. MOLECULAR SIZE OF THE ISOAGGLUTININS

Since the various anti-B isoagglutinins combine specifically with the same B agglutinogen groups, and since the thermal effect measured corresponds to all types of changes produced in the two reacting molecules, the possibility was considered that the differences between these isoagglutinins were not due, or not entirely due, to the specific parts of their molecules. This point is well illustrated by the study of the molecular dimensions (FILITTI-WURMSER *et al.*, 1953a). The measurements have been carried out on $\beta(A_1O)$, $\beta(A_1A_1)$ and $\beta(OO)$, with a Spinco ultracentrifuge, by the analytical method employing partition cells. The relative concentration of isoagglutinins in the two compartments (central and peripheral) was determined from the maximum number of B cells agglutinated at 4°C. The sedimentation constant is calculated from the formula given by TISELIUS *et al.* (1937),

$$S = -\frac{1}{2\omega^2 t} \ln\left(1 - \frac{2\Delta}{qxC}\right)$$

where ω is the angular velocity, t the time of centrifugation in seconds, C the initial isoagglutinin concentration, Δ the quantity of agglutinin which has passed from one compartment to the other, q the area of the surface of separation, and x its radial distance. In order to obtain the sedimentation constant at infinite dilution, measures were made first with sera at different dilutions in buffer, then with solutions of eluated agglutinins. The sedimentation constants measured are plotted against

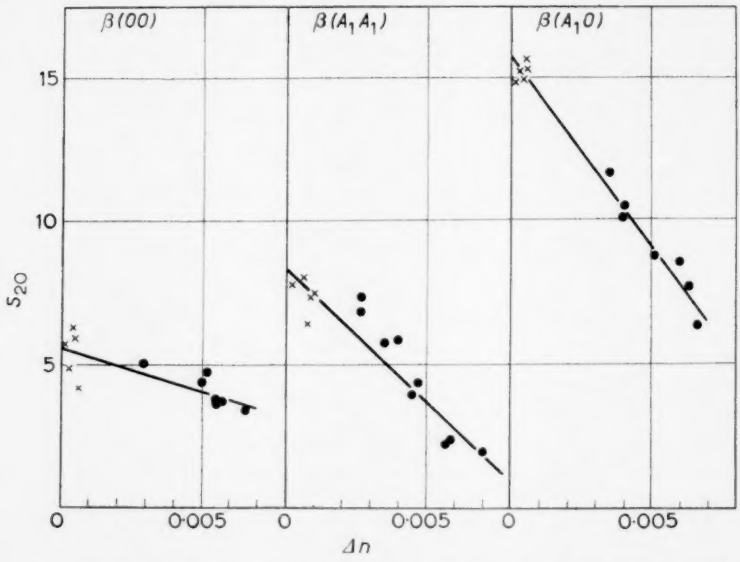

Fig. 5. Sedimentation constants, at 20°C, in Svedberg units, of three anti-B isohaemagglutinins in varying concentrations of total protein. Δn is the difference in refractive indices between buffer solution and diluted serum or eluate.

Δn which is the difference between the refractive indices of the isoagglutinin solution and the buffer. Fig. 5 shows the results of the experiments carried out on the sera of six A_1O individuals, six A_1A_1 individuals, and six OO individuals. Two series of measurements were made on OO sera in order to see whether the sedimentation velocity of β(OO) is influenced by the presence of anti-A isoagglutinins. In the first series, the OO sera contain anti-A isoagglutinins; in the second series the OO sera were deprived of anti-A isoagglutinins by previous absorption. The anti-A isoagglutinins have no influence on the value of the β(OO) constant of sedimentation.

The crosses situated near the axis of the ordinate (Fig. 5) represent the values recently observed (WURMSER *et al.*, 1954, 1956) with a solution of isoagglutinin obtained by elution from B stroma which were saturated with isoagglutinin. In these eluates there are 700 times less

of other proteins mixed with the isoagglutinins than in the original serum. The values of the sedimentation constants extrapolated to infinite dilution and at 20°C are respectively, in Svedberg units:

Isoagglutinins	S_{20}
$\beta(A_1O)$	15·7
$\beta(A_1A_1)$	8·4
$\beta(OO)$	5·7

The new values obtained with eluates, 15·7 for $\beta(A_1O)$ and 5·7 for $\beta(OO)$, agree with the results previously (FILITTI-WURMSER *et al.*, 1953a) reported, 15·5 and 6·5 respectively. The new value found for $\beta(A_1A_1)$ is 8·4 instead of 11. But nothing is changed as to the essential fact that the size of $\beta(A_1A_1)$ is definitely intermediate between the two others. These results seem to afford a rather strong confirmation of the idea that the differences found between the heats of reaction of the anti-B isoagglutinins of sera of different genotypes ought to be attributed to differences in the carriers of the specific groups and not to differences in the structure of the specific groups themselves.

One may perhaps explain the facts observed by AUBEL-LESURE (1955) in the same way; that is to say, by a modification of the molecules of isoagglutinin as a whole. In those experiments A_1O sera were treated for 45 to 60 minutes with glucose, within the concentration range $M/3$ and $M/18$, with a variation of temperature between 25°C and 37°C. The transformation after a period of induction is progressive. It leads to the irreversible formation of an agglutinin characterized by a value for $(-\Delta H)$ of approximately 40 kcal. The interpretation of these facts is by no means clear. However, a possible explanation may be a copolymerization of the isoagglutinin.

VI. Computations of Equilibrium Constants and Variations of Entropy

The evaluation of equilibrium constants requires, in addition to the red-cell counts which are sufficient for the determination of the heats of reaction, microdeterminations of nitrogen. Indeed the slopes of the straight lines which represent N_f/N_t as a function of $1/N_4'$ are inversely proportional to the product mK, and the number m can be obtained only if we are able to measure concentrations of agglutinin in absolute values. That is to say one needs to evaluate the value of the coefficient ϕ. This evaluation is based on the specific combination of the isoagglutinin of a serum with a known N_4 with the stroma of B cells. N_4' is determined on the supernatant and the nitrogen fixed on the stroma is measured using the micro-Kjeldahl method (JACQUOT-ARMAND and FILITTI-WURMSER, 1953). Table 4 gives examples of this

TABLE 4

Equivalence of the Concentration of Agglutinin expressed as N_4 with the Agglutinin Nitrogen Concentration

Volume of the mixture (ml)	*Stroma N (μg)*	*Combined agglutinin N (μg)*	*Combined agglutinin given by N_f × volume × (10^{-6})*	*Agglutinin in 1 ml serum when $N_4 = 1 \times 10^6$ N (μg)*
		$\beta(A_1O)$		
4	85·0	12·4	27,340	0·45
4	94·0	13·3	27,532	0·48
4	42·6	11·0	29,912	0·36
7	113·3	24·2	51,000	0·47
7	138·1	28·5	51,891	0·55
7	107·3	29·8	60,487	0·49
				Mean 0·47 ± 0·06
		$\beta(A_1A_1)$		
6	103·3	8·7	18,000	0·48
6	59·0	8·3	18,600	0·44
6	286·1	12·0	29,100	0·4
				Mean 0·44 ± 0·04
		$\beta(OO)$		
4	35·4	6·2	10,800	0·57
5·5	184·6	14·9	28,580	0·52
7	100·0	20·0	51,800	0·38
7	183·0	29·0	52,000	0·55
				Mean 0·50 ± 7

type of determinations for A_1O, A_1A_1 and OO sera. In the first column one finds the volume of the mixture of serum and stroma suspension, in the second column the nitrogen determined on the B stroma after agitation in the presence of an AB serum (in order to account for non-specific absorption); in the third column, the excess of nitrogen fixed on stroma when it is shaken in the presence of an anti-B serum; in the fourth column the quantity of fixed isoagglutinin expressed as $(N_4 - N_4') \times$ (volume in cubic millimetres). The last column lists the value R, that is the quantity of specific nitrogen, expressed in micrograms, contained in 1 ml of serum whose relative concentration of isoagglutinin is 1×10^6 expressed as N_4. The coefficient ϕ is equal to $M/(R \times 10^{-9} \times 6, 25)$, where M is the molecular weight of the agglutinin. One notes that, for a given quantity of stroma, when the quantity of isoagglutinin in the sera varies considerably, the R ratio remains constant. This fact is mentioned since the same value of R is used for the transformation of both the units of N_4 and N_4' to molarity.

In order to calculate ϕ it is necessary to know the molecular weight M of one isoagglutinin. No real estimation of the value of M can be made from the sedimentation constant without diffusion measurements. Nevertheless, as it is worth while to get at least approximate values of the equilibrium constants, probable values of M have been chosen, taking into account the sedimentation constants reported in the former section. The results of these computations are given in the following:

	M	ϕ
$\beta(A_1O)$	$\sim 500{,}000$	$1{\cdot}7 \times 10^{14}$
$\beta(A_1A_1)$	$\sim 200{,}000$	$0{\cdot}73 \times 10^{14}$
$\beta(OO)$	$\sim 125{,}000$	$0{\cdot}40 \times 10^{14}$

The molarity of each isoagglutinin in the different sera studied varied respectively:

$\beta(A_1O)$ from $1 \times 10^{-8}M$ to $6 \times 10^{-8}M$
$\beta(A_1A_1)$ from $1 \times 10^{-8}M$ to $3 \times 10^{-7}M$
$\beta(OO)$ from $2 \times 10^{-8}M$ to $3{\cdot}5 \times 10^{-7}M$

KABAT and BEZER (1945) have determined the nitrogen of human anti-B isoagglutinins fixed on B substance. According to their data when the agglutinin titer of the sera at 37°C is 1/4, the nitrogen antibody precipitated per millilitre of serum in microgrammes is 0·6 to 0·7. This data gives values of the same order of magnitude as those cited above for the concentration of anti-B isoagglutinin.

Evaluation of m. The straight lines such as those of Fig. 3 and Fig. 4 cannot be used for calculating m from the intercept on the ordinate. These lines represent measurements which have been made under conditions where only a small proportion of the agglutinogen groups is combined. It follows from this that $1/m$ falls at the origin of the axis in the graphic representation. For this reason one has to be content to evaluate the average number of agglutinogen groups per red cell under other experimental conditions.

When measured with a lower number of red cells, the N_f/N_t ratio will approach closer to being a measure of m. But in the experiments made with a small number of red cells and an elevated concentration of isoagglutinin, the difference between N_4 and N_4' is small and the results imprecise.

Only an approximate value of m is available. A first approximation of the value of m was obtained from experiments done with group O sera and B red cells at 4°C. The extrapolation gives a value for m greater than 1×10^5. In another set of experiments done with A_1O sera a value for m of 5×10^5 was found. The equilibrium constants quoted in Table 5 have been calculated with this value of m.

Using these equilibrium constants and the values of the heat of

TABLE 5

Thermodynamical Data for the Binding of anti-B Isohaemagglutinins by B Agglutinin

	$\beta(OO)$	$\beta(A_1A_1)$	$\beta(A_1O)$
$K_{37°}$ litre/mole	$0{\cdot}3 \times 10^7$	$0{\cdot}5 \times 10^7$	$0{\cdot}8 \times 10^7$
$K_{25°}$ litre/mole	$0{\cdot}3 \times 10^7$	$0{\cdot}8 \times 10^7$	$2{\cdot}2 \times 10^7$
$K_{4°}$ litre/mole	$0{\cdot}4 \times 10^7$	$2{\cdot}2 \times 10^7$	$17{\cdot}0 \times 10^7$
$\Delta F_{37°}$ kcal/mole	9·2	9·5	9·8
$\Delta S_{37°}$ cal/degree	+ 24	+ 9·7	− 20

reaction from Table 3, one may calculate the corresponding variation of entropy ΔS by mean of relationship (5):

$$\Delta S = (\Delta H + RT \ln K)/T$$

Table 5 indicates the results obtained for three isoagglutinins: $\beta(A_1O)$, $\beta(A_1A_1)$ and $\beta(OO)$.

The most serious source of uncertainty in these calculations is derived from the determination of the value m. However, this uncertainty does not affect the characteristic differences between the variations of entropy corresponding to the fixation of three kinds of isoagglutinins on B agglutinogen.

The significance of these very great differences between the values of ΔS corresponding to the combination of $\beta(A_1O)$, $\beta(A_1A_1)$ and $\beta(OO)$ with the same B agglutinogen is worth consideration. The respective molecular dimensions of these isoagglutinins do not explain entirely the differences of ΔS if one only considers the contribution of the terms of translation and rotation of the molecule as a whole in the expression of affinity. The ratio of the partition functions for $\beta(A_1O)$ and for $\beta(OO)$ is approximately $(M_{A_1O}/M_{OO})^4$, where M_{A_1O} and M_{OO} are the molecular weights of the molecules, supposedly spherical and with equal densities. (M_{A_1O}/M_{OO}) is almost equal to 4. Therefore one has a difference of free energy $RT \ln 4^4$, of approximately 3·4 kcal, which corresponds to a variation of entropy of 11 units, whereas the variation between ΔS_{OO} and ΔS_{A_1O} approaches 44 units. But the differences between the carriers of the anti-B group are able to contribute to the differences of affinity in ways other than by their translational and rotational terms. It is not necessary to suppose that the specific sites differ in the nature or arrangement of the aminoacids involved in the linkages on $\beta(A_1O)$, $\beta(A_1A_1)$ and $\beta(OO)$. Indeed, one can cite many examples of the combination of a specific group on a protein molecule having an effect through the whole molecule. The $\beta(OO)$ isoagglutinin whose fixation is without a great thermal effect combines with the B agglutinogen group because of the increase in entropy. This increase

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may perhaps be due to a type of partial denaturation of $\beta(OO)$ following the "strain" produced at the specific site. An intramolecular perturbation would be brought about also in the case of $\beta(A_1O)$ isoagglutinin, but the carriers are so different for the two agglutinins that one can easily suppose some rearrangement with formation of several supplementary hydrogen bonds for $\beta(A_1O)$. Obviously the same assumption may be made for $\beta(A_1A_1)$.

VII. Homogeneity of the Isohaemagglutinins

There are many proofs particularly from the work of Heidelberger and his school (Heidelberger and Kendall, 1935; Kabat and Heidelberger, 1937) that there exist antibodies with different reactivities towards one determinant group and antisera with varying proportions of these antibodies. This nonuniformity appears in some experiments (Filitti-Wurmser and Aubel-Lesure, unpublished) which have been made on an anti-B serum of rabbit purified by absorption on A_1O and O cells. The value of the product $(N_t/N_f)N_4'$ varies from 2 to 4·5 at 37°C instead of being constant as with the isohaemagglutinins.* In contrast, one cannot help but be impressed by the fact that all the normal human sera of the same genotype when mixed with B red cells gives values of N_t/N_f which when plotted against $1/N_4'$ fall on the same line. This fact would obviously be difficult to explain if each individual serum was a mixture of anti-B agglutinins. Moreover, it is unlikely that all sera of the same genotype possess a constant proportion of different isoagglutinins.

We shall now consider the quantitative aspect of the question. In view of this discussion it is advantageous to treat the experimental results described above in a somewhat different manner. In all the experiments the number of G groups combined is small with respect to the total number of G groups on the red cells present. The possibility therefore follows of assuming N_t as a value proportional to the concentration (G) of the free groups. The ratio r_A of the combined isoagglutinin to the total isoagglutinin is equal to N_f/N_4. Thus, if the isoagglutinin is univalent, or react like it is univalent for the reasons already indicated, one has:

$$r_A = \frac{N_f}{N_4} \approx \frac{K(mN_t/6 \times 10^{17})}{1 + K(mN_t/6 \times 10^{17})} \qquad \ldots\ldots(6)$$

It is convenient here to represent the experimental results by putting this relationship in the form:

$$\frac{N_f}{N_4} \times \frac{6 \times 10^{17}}{mN_t} = K - K\frac{N_f}{N_4} \qquad \ldots\ldots(7)$$

* According to equation (3) the constancy of $(N_t/N_f)N_4'$ holds in the range of experimental values of N_4', that is for $N_4' \ll \phi/K$.

As SCATCHARD (1949) has emphasized, this type of representation has the advantage of not minimizing the range of concentration where the experimental data are lacking. The values of (N_f/N_4) $(6 \times 10^{17}/N_t)$ are plotted against the values (N_f/N_4). The results at 37°C are given in Fig. 6. One notes that the plots representing the measurements

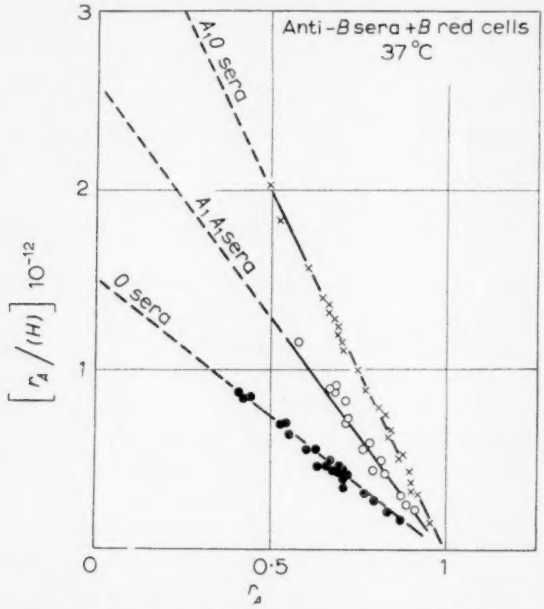

Fig. 6. Linear plots of binding data for anti-B isohaemagglutinins according to equation (7). Abscissa: fraction of combined agglutinin. Ordinate: fraction of combined agglutinin divided by red-cell molar concentration (H). In the zone corresponding to dashed lines, homogeneity is tested by elutions and successive absorptions.

with $\beta(A_1O)$ and $\beta(OO)$ attain or pass the abscissa 0·5 without deviating from linearity. However, in this test of equation (7) there exists a large gap. The possibility is not excluded that measurements made with an extremely small number of red cells, corresponding to a small value of (N_f/N_4), would deviate from linearity.

With this problem in mind the study of eluted isoagglutinins give additional information. Table 6 indicates the results obtained after absorbing 90 per cent of $\beta(A_1O)$ isoagglutinin on stroma of B red cells at 4°C. The elution at 52°C frees 32 per cent of the fixed $\beta(A_1O)$. An aliquot of the solution with eluted $\beta(A_1O)$ is shaken with B red cells. The values of the product $(N_t/N_f)N_4'$ at 37°C and 25°C agree with the values normally obtained (Fig. 4) on total sera for $\beta(A_1O)$. Thus, the elution has not liberated a fraction with a lower affinity. Analogous results are obtained with $\beta'(A_1A_1)$ isoagglutinin.

TABLE 6

Equilibrium Measurements between B *Red Cells and Eluates of* $\beta(A_1O)$ *and* $\beta(A_1A_1)$

I. 91 per cent of $\beta(A_1O)$ contained in a serum is fixed on B stroma at 4°C and 32 per cent of the fixed $\beta(A_1O)$ is eluted at 52°C.

II. 76 per cent of $\beta(A_1A_1)$ contained in a serum is fixed on B stroma at 4°C and 33 per cent is eluted at 52°C.

$N_t \times 10^{-3}$	$N_4' \times 10^{-3}$	$N_f \times 10^{-3}$	$(N_t/N_f)N_4' \times 10^{-5}$
	I. at 37°C		
793	127	673	1·49
	I. at 25°C		
480	90	710	0·60
	II. at 37°C		
300	210	290	2·16

It is also observed that the isoagglutinin remaining free, when serum is mixed with B cells and shaken, has the same affinity as the isoagglutinin in the initial serum. If an agglutinin with a weak affinity did exist in sufficient quantity in the serum, it would predominate among the isoagglutinin not fixed in the first agglutination. Table 7 presents the results of successive absorptions on B red cells of anti-B sera from individuals of different genotypes. One sees that the values

TABLE 7

Equilibrium at 37°*C in a Mixture of* B *Red Cells with Serum Untreated* S; *with Supernatant after a First Agglutination with* B *Red Cells at* 37°*C* S_B; *with Supernatant after Absorption on Opossum Red Cells at* 4°*C* S_{op}

	$N_t \times 10^{-3}$	$N_4' \times 10^{-3}$	$N_f \times 10^{-3}$	$(N_t/N_f)N_4' \times 10^{-5}$
		A_1O		
S	1390	124	1116	1·53
S	334	380	860	1·47
S_B	346	130	295	1·51
S_{op}	1123	77	553	1·60
S_{op}	340	430	915	1·56
		A_1A_1		
S	450	625	1335	2·11
S_B	450	130	286	2·01
		OO		
S	450	1220	1380	3·98
S_B	450	370	440	3·78
S_{op}	316	400	325	3·90
S_{op}	905	166	350	4·29

of $(N_t/N_f)N_4'$ are the same for the initial S serum and for the S_B supernatant obtained by centrifugation after the first agglutination. For example, for $\beta(A_1A_1)$ at 37°C, if 68 per cent of this isoagglutinin is fixed on $4{\cdot}5 \times 10^5$ B red cells per cubic millimetre in the first agglutination, in the second agglutination carried out on the non-fixed $\beta(A_1A_1)$, again in the presence of $4{\cdot}5 \times 10^5$ B red cells per cubic millimetre, 68 per cent of $\beta(A_1A_1)$ is fixed.

Moreover, experiments made with mixtures of various sera, some of which will be described in Section IX, allow one to consider $\beta(A_1O)$, $\beta(A_1A_1)$ and $\beta(OO)$ as homogeneous within the experimental error.

Finally it is necessary to emphasize that the homogeneity of anti-B isohaemagglutinins discussed here concern their affinity with respect to the B agglutinogen, and not with respect to every other antigen. The question was raised by a recent paper of Owen (1954), who extended the investigations of Friedenreich and With (1933). He found that some human anti-B sera retain, after several absorptions on opossum red cells, an agglutinating power with respect to B red cells, while other anti-B sera lose nearly all their agglutinating power. Owen sees in these experiments the confirmation of the idea that there exists in anti-B sera different agglutinins, some absorbable and others non-absorbable by an antigen of the opossum red cells. The interpretation of these facts is less simple. Jacquot-Armand *et al.* (1956), studying this phenomenon, have found that the more or less important absorption of the anti-B isoagglutinin by the opossum red cells depends on the proportion of anti-opossum agglutinin present in the serum. All human sera, including the AB sera, which do not contain any antihuman isoagglutinin, contain antiopossum red cells agglutinins. The fact that the antiopossum red-cell agglutinin is able to exist in different quantities in diverse human sera may explain the variable proportions of human anti-B absorbed on opossum cells. In any case it has been practically impossible to absorb on the opossum red cells all of the $\beta(A_1O)$, $\beta(A_1A_1)$, $\beta(A_2O)$ and $\beta(OO)$ of the human sera studied. It was thus necessary to investigate whether the fraction of anti-B which was not absorbed by the opossum cells has an abnormal affinity with respect to B antigen. Here again it was observed that the value of $(N_t/N_f)N_4'$ is the same for the isoagglutinin not fixed on animal cells as the value $(N_t/N_f)N_4'$ in the original serum (Table 7).

To summarize, it has been impossible to find in a serum with a determined genotype a heterogeneity of the isohaemagglutinin which manifests itself by a difference in affinity for B antigen. It is granted that differences in affinity could exist which are not detected by the sensitivity of the method employed. An estimation of this possibility would be rather intricate since it would depend both on the quantity of hypothetical components and on the affinity that one would suppose

for each of them. We can say that the sensitivity of the method as it stands is sufficient for distinguishing 10 per cent of β(OO) mixed with 90 per cent $\beta(A_1O)$.

VIII. Agglutinogen Groups

In comparing various agglutinins, the heterogeneity of the red cells is not to be taken into consideration, for in the very large number of experiments described above the same red-cell suspension was used to prepare the mixtures with the various sera.

However, it is noteworthy that the measurements performed over a period of ten years involve a large number of samples of B red-cell suspensions, from many different donors. Thus to the homogeneity of the anti-B sera apparently corresponds a similar homogeneity of the B red cells. We cannot estimate the degree of the latter because, as already emphasized, the proportion of agglutinogen groups combined with agglutinin molecules was always very small.

The fact that no difference in the product $(N_t/N_f)N_4'$ was observed when AB cells were substituted for BO cells indicates that, within the precision of the method, the product mK is the same for these two types of red cells. However it appears from the recent experiments of Wilkie and Becker (1955b) that there is a difference in the number of B groups carried by AB and BB red cells.

IX. Formation of the Isohaemagglutinins

Let us now consider the mechanism of formation of isohaemagglutinins. There exist essentially two conceptions concerning their origin. Either the isohaemagglutinins result from an immunization by the environment, or their origin is genetically determined by a pleiotropic gene. For the case of anti-B, on the first hypothesis, both anti-A and anti-B are produced by all individuals, but antigen B present in the individuals B removes anti-B. On the second hypothesis, a single gene directly causes to occur both the antigen A in the red cells and antibody anti-B in the serum.

The differences observed among the various anti-B isohaemagglutinins fit more simply the latter hypothesis, but do not necessarily contradict the former if only the formation of the specific anti-B group is considered. Indeed, the imprint of the genotype may be carried by the precursor of the isohaemagglutinin, and the pleiotropic effect restricted to the formation of this protein.

In the current conception of Breinl and Haurowitz (1930), antibodies are globulin molecules with surfaces complementary to the specific configuration of the antigen. The complementarity is assumed to be brought about by a definite kind of folding of the peptide chain of the precursor. The characteristic feature of the isohaemagglutinin

formed by an individual of a given genotype would thus be derived from that of the precursor which gives rise to this agglutinin. It would be interesting to see if a "genotypic imprint" is found in antibodies of experimental or infectious origin.

We have to consider now the problem which arises from the occurrence of an anti-B agglutinin $\beta(A_1O)$ differing from $\beta(A_1A_1)$ and $\beta(OO)$ by its affinity and its molecular size (WURMSER and FILITTI-WURMSER, 1953).

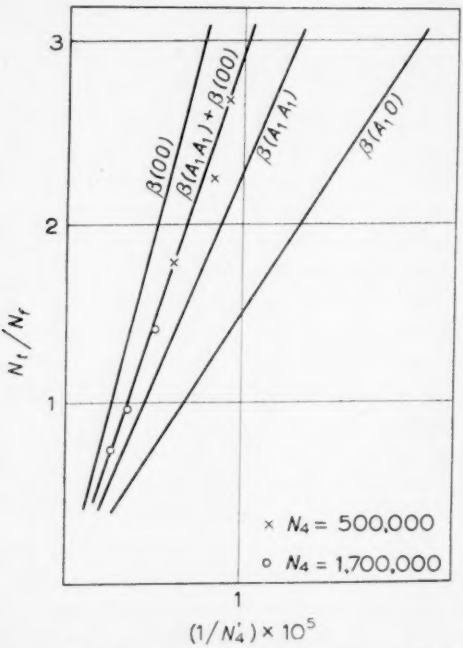

Fig. 7. Comparison of $\beta(A_1O)$ agglutinin and a mixture of $\beta(A_1A_1)$ and $\beta(OO)$ with respect to their affinity for B agglutinogen. Same abscissa and ordinate as in Fig. 3. (From FILITTI-WURMSER *et al.*, 1953b.)

It may be argued that we are dealing with a mixture of $\beta(A_1A_1)$ and $\beta(OO)$ agglutinins. But the study of mixtures of group O and subgroup A_1A_1 sera showed (FILITTI-WURMSER *et al.*, 1953b) that this objection is not valid. The values of the ratio N_t/N_f have been calculated for such mixtures as a function of N_4' by applying the mass action law and utilizing the equilibrium constants of Table 5. The results have been compared with the experimental determinations (Fig. 7) and a satisfactory agreement was obtained. The experimental points fit the calculated curve. In the region of quasi-linearity, the slope at 37°C is equal to 3×10^5. It is intermediary between the slopes which correspond to the $\beta(A_1A_1)$ and $\beta(OO)$ isohaemagglutinins which are respectively $2{\cdot}3 \times 10^5$ and $4{\cdot}0 \times 10^5$. Therefore the $\beta(A_1O)$ isoagglutinin, which

gives a slope of $1{\cdot}5 \times 10^5$, cannot be confused with a mixture of $\beta(A_1A_1)$ and $\beta(OO)$.

The isoagglutinin $\beta(A_1O)$ is an example of a protein characteristic of a heterozygote. Very few hybrid substances have been detected until now. Their possible significance for the explanation of heterosis has been suggested by HALDANE (1955). Thus the occurrence of $\beta(A_1O)$ is of very great interest.

From the standpoint of the mechanism of its formation, the situation is entirely different from that of haemoglobins found in sicklaemic individuals. In the heterozygotes, as PAULING *et al.* (1949) have shown, both the normal haemoglobin and the particular sicklaemia haemoglobin are present. This is explained by a competition between the normal gene and its allele. In the case of the $\beta(A_1O)$ isohaemagglutinin, how can the co-operation of the two alleles in its formation be explained? To give an idea of the possible mechanisms, let us return to the hypothesis that the synthesis of the isoagglutinin is carried out in two stages. These would be, first, the formation of the precursor, and, second, the acquisition by this precursor of the particular configuration which will permit it to react with the specific group of agglutinogen B. Each of the A_1 and O genes presides over the synthesis of a precursor. But when the A_1 and O genes are present at the same site of formation, the two precursors formed are later associated to produce a hybrid precursor.

Work which has a particular bearing upon this problem must be mentioned at this point. DUNSFORD *et al.* (1953) made a very complete study of the first and only known case of a human being, originally of group O, but having two types of red cells: 61 per cent of them are group O and 39 per cent are A_1. This individual is a "chimera" whose twin brother, who died 25 years ago, as established by the authors, was subgroup A_1.

FILITTI-WURMSER and JACQUOT-ARMAND (1956) have investigated the anti-B agglutinin of the serum of this chimera. They found that it is $\beta(A_1O)$. This is consistent with the hypothesis outlined above.

X. CONCLUSIONS

In conclusion it may be said that the fixation of isohaemagglutinins on red cells obeys the mass-action law. This made it possible to obtain quantitative data informing us about, for instance, the heat of reaction and the entropy change. Unexpected facts which appeared from these data and which were verified by an entirely independent method have proved their usefulness for characterization problems. For instance, it was found that, among isohaemagglutinins specific against the same agglutinogen, differences exist which are themselves specific. A mark is superimposed which depends on the genotype of the individual who

synthesized the agglutinin. These differences, revealed by thermodynamic properties, have been confirmed by measurements of molecular size.

One of the experimental facts which emerged from these studies is the occurrence of an agglutinin synthesized by heterozygous individuals and different from that made by either homozygote. Many problems appear in this connection, all of which are of interest for general biology.

The results just summarized have been obtained by studying the molecule of agglutinin as a whole. But without doubt further research along the lines described will yield a great deal of information about the structure and properties of the groups involved in the binding with agglutinogens.

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USE OF LABELLED PLASMA PROTEINS IN THE STUDY OF NUTRITIONAL PROBLEMS

A. S. McFarlane

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3

USE OF LABELLED PLASMA PROTEINS IN THE STUDY OF NUTRITIONAL PROBLEMS

A. S. McFarlane

THE realization that plasma proteins play an important role in the day-to-day metabolism of higher animals we owe mainly to the work of G. H. WHIPPLE and his numerous collaborators in the decade 1935/45. Their technical approach was of a classical nutritional type which will be summarized briefly below. Towards the end of this period the isotopic method was introduced by SCHOENHEIMER and RITTENBERG and taken up by numerous investigators including WHIPPLE and members of his group. The results have not been easy to reconcile with earlier ones, and part of this essay is to be regarded as an attempt to do this. The remainder is devoted to some less directly nutritional aspects of the behaviour of plasma proteins as observed with the use of labelled molecules. No apology is necessary for presenting mainly physiological topics of these kinds in a biophysical volume since the views discussed are derived largely from the use of a new and complicated physical technique and depend critically on the interpretations which may be given to measurements made with its help.

I. NUTRITIONAL STUDIES

The focus of attention by WHIPPLE and others (cf. MADDEN and WHIPPLE, 1940; MELNICK *et al.*, 1936a,b; WEECH, 1939) has been on the liver as the main, if not the sole, organ for making and storing plasma proteins including albumin, globulins, fibrinogen, and the globin of haemoglobin. This view is mainly derived from the following observations. Healthy dogs on a generous protein diet were able to maintain an almost constant plasma protein level (6 g/100 ml) in spite of daily withdrawals of as much as a quarter of their circulating plasma. The technique of withdrawal which involves return of red cells to prevent anaemia developing, they have called plasmapheresis. Only when the intake of protein in the diet was limited did the plasma protein level begin to fall, but even in such cases healthy dogs were able to stabilize it at a lower level. On a so-called basal protein diet the plasma protein level remained constant indefinitely at 4·0 g/100 ml, while 1-g plasma protein per kilogramme body weight was being withdrawn daily.

In this state of physiological strain the equilibrium achieved is inevitably a delicate one susceptible to a variety of influences and

especially to changes in the quality or quantity of dietary protein. When most favourable protein (beef serum) was fed in moderate amounts, the highest recorded efficiency of conversion of dietary nitrogen to plasma protein nitrogen resulted. This was placed variously at 38 per cent by MADDEN and WHIPPLE (1940) and 53 per cent by MELNICK *et al.* (1936), according to different methods of calculation, and could be increased if the animal was made anaemic as well as hypoproteinaemic (ROBSCHEIT-ROBBINS *et al.*, 1945). A noteworthy feature was that considerably more than 1-g plasma protein per kilogramme had to be withdrawn daily for the first 7 to 10 days before the animals settled down to deliver this amount regularly. This was taken to mean that there exists outside the circulation a reservoir of plasma protein which must first be reduced or even completely drained before a new equilibrium state can be established.

If liver function was intentionally impaired, for example by injecting acacia which accumulated in liver cells, or if the portal blood was diverted from the liver by an Eck fistula, the ability to manufacture plasma protein under the dual stresses of anaemia and hypoproteinaemia was much reduced—in the case of Eck fistula dogs to as little as one-tenth or 0·1 mg/kg/day. This was believed by WHIPPLE *et al.* (1945) to show that the liver is the critical organ for production of blood proteins, including the globin of haemoglobin. This view is supported in their opinion by a mass of clinical evidence showing that diseases affecting the liver, such as cirrhosis and hepatitis, are associated with low levels of plasma albumin, prothrombin and fibrinogen. On the other hand such conditions are not usually associated with anaemia, a fact which seems to have escaped the attention it probably deserves in relation to the metabolic origin of globin.

In a different type of nutritional experiment WHIPPLE and his collaborators examined the ability of plasma protein and haemoglobin administered parenterally to meet the nitrogen requirements of dogs on a nitrogen-free diet. HOLLMAN *et al.* (1934) established that daily injections of plasma will keep dogs in nitrogen balance and in good health for 20 days or more and without an increase in plasma-free amino-acid levels occurring (HOWLAND and HAWKINS, 1938). Thus injected plasma proteins appear to be used directly to meet the nutritional needs of the animal, i.e. without prior breakdown to free amino acids. Haemoglobin given intraperitoneally by MILLER *et al.* (1945) was not used so efficiently for this purpose as were plasma proteins, but in larger amounts it maintained the animal in nitrogen balance. These authors state "it is surprising that an incomplete protein (globin) can contribute so effectively to the protein pool as it must obviously be supplemented by amino acids inadequately represented in globin drawn from some reservoir store to produce cell protein

or plasma protein. The whole globin in some dogs is more effectively used than the haemoglobin digests, suggesting that some of the globin may be used without much breakdown and loss of nitrogen as is so conspicuous in the body use of plasma proteins where there is no loss of nitrogen."

From experiments of the foregoing types the thesis is developed that a mobile pool of proteins exists inside and outside the vascular system which can be used in emergency for a variety of purposes including tissue repair and red cell formation. The size of the pool is variable, depending on the previous dietary history of the animal, but with an upper value estimated by MADDEN *et al.* (1938) to be equivalent to twice the mass of protein in the circulating plasma. Prolonged fasting with or without plasma withdrawals depletes the pool without apparently causing permanent injury to the mechanism for forming plasma proteins. A minimum period of 2–6 weeks of continuous moderate withdrawals on a basal diet is necessary to deplete this reserve store, heavier withdrawals over a shorter period being ineffective. Large amounts of protein given in a single meal to a depleted dog have been shown by MADDEN *et al.* (1937) to lead to increased plasma-protein production lasting over the next 14 days. This carry-over effect of a diet presents a major technical difficulty in interpreting the results of many depletion experiments, opinions differing sharply on precisely how long a period of preliminary plasmapheresis is necessary before a new state of nutritional equilibrium can be achieved (cf. MADDEN and WHIPPLE, 1940; MELNICK *et al.*, 1936a,b).

It is important also that no unsuspected accessory factor or critical amino acid should be in short supply. For example ROBSCHEIT-ROBBINS *et al.* (1945) show that lack of iron or isoleucine can severely limit haemoglobin production and lead to erroneous deductions concerning the animal's basic capacity to produce this protein. Within these limitations the nutritional methods of investigation outlined here have given important information about plasma-protein production, but clearly the information is derived from animals in more or less serious states of nutritional strain, a qualification which does not apply to information derived from the use of isotopes.

II. In Vivo Synthesis of Plasma Proteins from Labelled Amino Acids

Following the pioneer work of SCHOENHEIMER, RITTENBERG, and their collaborators, who fed ^{15}N-labelled amino acids to rats over a period of days and observed the rapid appearance and subsequent disappearance of labelled plasma proteins (cf. reviews by SCHOENHEIMER, 1942; RITTENBERG and SHEMIN, 1946; SPRINSON, 1948), FINK *et al.* (1944) fed or injected [ε-^{15}N] lysine to dogs. Later MILLER *et al.* (1949)

made a more detailed study of the appearance of labelled plasma proteins in the dog following the feeding of *DL*-[ε-^{14}C] lysine at a single meal. At 24 hr, when specific activities of plasma proteins had maximum values, the specific activity of total plasma protein was 20 times that of haemoglobin. Globulin specific activity reached a maximum which was more than twice that of albumin, and subsequently declined faster until the two proteins had almost the same value at 70 days. At 24 hr, 1·5 per cent of the label was associated with proteins in the plasma while activity in expired CO_2 rose and fell in parallel with nonprotein ^{14}C activity in the plasma. The choice of lysine for these experiments was prompted by the fact that this amino acid was shown by FOSTER, SCHOENHEIMER and RITTENBERG (1939) to be practically unique in resisting deamination *in vivo*. The low utilization (1·5 per cent) was partly due to the use of the DL acid, the unnatural D form being unused, and partly to feeding large amounts of inactive protein with it.

Proteins of the viscera, intestine and duodenum of dogs killed at 24 hr were found to be highly labelled, as was also observed by BORSOOK *et al.* (1950). Significant secondary labelling of glutamic and aspartic acids and of arginine took place as the experiment progressed. The authors fully realized that the labelled lysine and other amino acids labelled secondarily from it were being reused, and therefore that rates of decline of plasma protein specific activities did not directly reflect rates of protein metabolic breakdown. They deduced that globulin is metabolized faster than albumin in the dog, and therefore that this animal should be better able to replace globulin than albumin after plasmapheresis, as indeed they had already shown to be the case. YUILE *et al.* (1952) fed *L*-[^{14}C] lysine or plasma proteins labelled with this amino acid to dogs and observed similar rates of appearance of the label in plasma, urine, and expired air in both types of experiment. They concluded that the orally administered protein was completely broken down. Maximum labelling of plasma proteins occurred at 7 hr, the activity in plasma proteins at this time representing as much as 6 to 8 per cent of the activity fed. Globulin again became labelled to a higher maximum value than albumin, but received proportionately less of the label than did albumin when the active protein was accompanied by substantial amounts of inactive protein in the diet. Expired CO_2 had maximum activity at 1 to 2 hr corresponding to a breakdown rate of 4 per cent of the dietary labelled protein per hour. However, this rate fell sharply, as did also the nonprotein-bound activity of the plasma, and after 7 hr these two and the specific activity of plasma protein declined in parallel. Combined urinary and faecal excretions were low throughout—less than 1 per cent of the dose daily—whereas total expired $^{14}CO_2$ after 48 hr represented 16 to

28 per cent, the remainder being present as labelled protein in the carcass.

ABDOU and TARVER (1952) obtained essentially similar results after oral administration of [^{14}C] serine-labelled protein or of ^{14}C-labelled *Rhodospirillum* organisms to rats. TARVER and REINHARDT (1947) also fed [^{35}S] methionine to hepatectomized dogs and showed that labelling of tissue proteins proceeded at the same rate as in normal dogs. Much work with isolated organs and tissue slices which is reviewed by TARVER (1954) confirms that cells in many parts of the body are able to build protein from amino acids without help from the liver. Thus we must agree with SCHOENHEIMER's view that there is keen competition at all times for injected labelled amino acids. However, they are not used solely for protein-forming purposes, as is indicated by the high initial rate of expiry of $^{14}CO_2$. The curve of free amino acid specific activity should therefore fall away rapidly with time, but not necessarily in parallel with the curve of plasma nonprotein ^{14}C activity. BARRY (1952) measured free amino specific activities after injecting [carboxy-^{14}C] lysine and similarly labelled tyrosine into lactating goats, and found these at 2 hr to be less than 10 per cent of the values at 20 min: similar results have been obtained in rabbits using [^{14}C] glycine (DOVEY *et al.*, 1954; MCFARLANE, 1952).

II. 1. *Measurement of free amino-acid specific activities*

The precise course of free amino-acid specific activities must have an important bearing on the appearance of labelled proteins in the plasma, and it is unfortunate therefore that formidable technical difficulties are encountered in obtaining the necessary data. These arise from the small amounts of free amino acids available in plasma samples and from the presence of other labelled substances which are difficult to remove. Barry's values obtained with large plasma samples are probably the most accurate so far obtained. He specifically liberated the carboxyl carbon of the amino acids in protein-free filtrates by means of lysine and tyrosine decarboxylases. Unfortunately he made no measurements after 12 hr, and did not measure specific activities of individual plasma proteins. Any uncertainty in his measurements is probably concerned with the precise ratio of carrier amino acid, which he was obliged to add, to the amount of labelled amino acid in the diffusate.

The [^{14}C] glycine measurements referred to in the rabbit were made over the full period of a plasma protein turnover experiment, but in addition to uncertainty regarding the proportion of glycine in rabbit free amino acids (which was assumed to be constant throughout) allowance could not be made for secondary spreading of the label, e.g. to serine. Both difficulties can be partly overcome by using a repre-

sentative mixture of labelled amino acids and by assuming that spreading of the label from one amino acid to another as the experiment progresses is a mutually compensatory process over the whole pool. This assumption is clearly an approximation, but fortunately for it those amino acids which tend preferentially to gain in activity by secondary

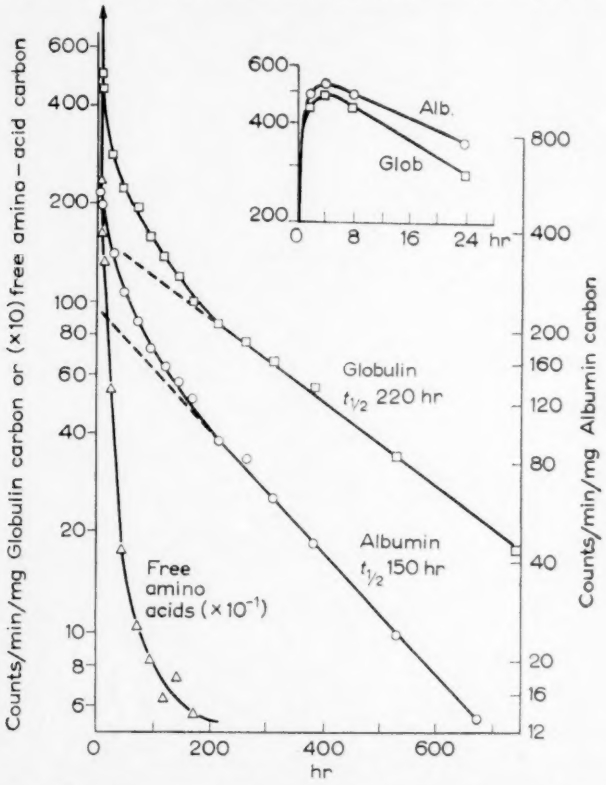

Fig. 1. Specific radioactivities of free amino acid and protein carbon of plasma after injecting a hydrolysate of ^{14}C-*Chlorella* protein into rats. Amino-acid values are maximum ones since all diffusible radioactivity of plasma is attributed to amino acids (cf. text).

labelling (glutamic and aspartic acids and alanine) by virtue of their close contact with the tricarboxylic-acid cycle also tend to lose activity rapidly into the large metabolic pool represented by the cycle. If the assumption is correct it is not necessary to know the concentration and carbon content of individual amino acids in the mixture, but only the mean total values.

Campbell *et al.* (1956a) injected hydrolysed ^{14}C-*Chlorella* protein (cf. Catch, 1954) into normal rats (0·05 mg/300 g rat) and followed specific activities of albumin, globulin, and free amino acids for 3 weeks (Fig. 1).

Albumin and globulin attained maximum values in 2 to 4 hr, at which time 2·0 per cent of the injected activity was associated with proteins in the plasma. Thereafter specific activities of both declined rapidly until, at the 4th day in the case of albumin and the 8th day in the case of globulin, truly exponential decay rates were established. Plasma samples were dialysed and total activities in protein-free

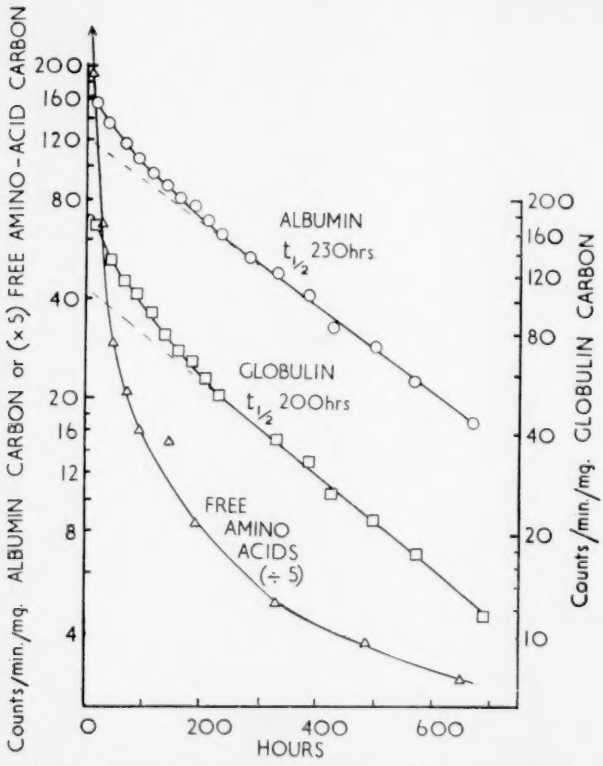

Fig. 2. Information as in Fig. 1 but for rabbit instead of rat.

diffusates measured and expressed per milligramme amino-acid carbon. Activity present in diffusable substances other than amino acids was thus attributed to the latter so that values obtained are to be regarded as *maximum* and not actual amino-acid values. Similar measurements have been made after injecting ^{14}C-protein hydrolysates into rabbits (Fig. 2). MILLER, BLY and BALE (1954) obtained a curve of non-protein activity after giving [^{14}C] lysine to rats which is essentially the same as the amino-acid curve in Fig. 1.

Decline of maximum amino-acid specific activities both in the rat and rabbit is so rapid in the first few minutes that it is impossible to measure the initial values with any precision. Estimates may be

obtained however by assuming the injected amino acids to be distributed throughout a pool equal to all the free amino acids of lymph and plasma. Measured values at 2 hr were small percentages of these estimated initial values—less than 1 per cent in the case of the rabbit and 0·25 per cent in the case of the rat. Inside 5 min more than 90 per cent of the injected activity had left the lymph and plasma. This amply confirms the findings of Borsook *et al.* (1950) in mice, findings which were open to question on the grounds that large amounts of glycine, histidine, leucine, or lysine were injected (up to thirty times the amount of the same amino acid in the plasma). Since the weights of *Chlorella* protein hydrolysate used in these experiments with rats and rabbits were less than 1 per cent of the free amino acids in the recipients' plasma, it is evident that the rapid disappearance of labelled amino acids did not result from raising the free amino-acid level, but represents a true exchange of amino acids proceeding in both directions.

Both rat and rabbit results show that an amount of amino acids equivalent to all the free amino acids of lymph and plasma are exchanged at least every 5 min. In metabolic terms this is a massive exchange and may indeed make this one of the dominating metabolic processes in the body. For instance, the weight of amino acids in lymph and plasma is about 1 per cent of the weight of plasma proteins, so that this rate of amino-acid exchange may be regarded as equivalent to a turnover rate for plasma proteins of 1 per cent in 5 min. Rabbit total plasma protein is normally made and replaced at approximately 8 per cent per day or only one-fortieth this rate. Thus the rapid disappearance of amino acids is not required to any major extent for plasma protein production, and indeed this could not be so for the additional reason that the activity which disappeared in 10 min from lymph and plasma was found at biopsy by Borsook and co-workers (1950) to be diffusely distributed in the viscera and gastrointestinal tract, i.e. without exceptional localization in the liver as the seat of albumin synthesis.

II. 2. *Labelling of visceral proteins*

Some of the activity found in the viscera must of course be present as free intracellular amino acids, but for the whole of it to be present in this form it would be necessary to postulate improbably high concentrations or specific activities of free intracellular amino acids. All the evidence suggests that most of the activity is associated with intracellular protein which is in process of rapid exchange with free amino acids. Results of many experiments with perfused organs and tissue slices suggest almost equally rapid rates of protein production (Green and Anker, 1955; Askonas and Humphrey, 1955) labelled proteins

being found in significant amounts within 10 min of presenting the organ or tissue with labelled amino acids. Labelled plasma proteins are usually detectable in the blood stream within 15 min of injecting the amino acid, and the production of labelled casein in milk is shown by CAMPBELL and WORK (1952) and by BARRY (1952) to precede the appearance of labelled plasma proteins in the same animal. Against this background the high rate of synthesis of labelled intracellular proteins by viscera may not appear so remarkable.

Thus isotope experiments on animals in protein and amino-acid equilibrium demonstrate the existence of a large intracellular protein pool which is potentially highly mobile in a metabolic sense, since it is in rapid reversible exchange with plasma-free amino acids. This protein is probably not of a particular type but comprises many kinds with different turnover rates. For practical purposes of course all the protein of the body is "mobile" in this metabolic sense, with the major proportion in muscle turning over extremely slowly.

Thus we may visualize plasma-free amino acids as constituting a central pool communicating reversibly with numerous protein pools. A sudden depletion of protein or amino acids occurring in any one pool will be made good by migration from all the others. At first the loss will be borne mainly by the compartment in most rapid exchange with the central one. Redistribution will then continue in all compartments until ultimately the loss is evenly distributed in proportion to pool size. Therefore in considering the problem of plasma protein loss by plasmapheresis a critical consideration is whether there exists a pool of protein in the body which is in more rapid exchange with the central one than the plasma protein pool, and which is of large enough capacity to take care of the emergency. The shapes of free amino-acid specific-activity curves show that the central pool is turned over many times in the first hour at rates which are higher than those required for plasma protein production. This indicates that there is indeed such a pool and one which is theoretically adequate to take care of protein withdrawals at rates up to 2 or 3 times that of normal plasma protein production. WHIPPLE and his collaborators found that regular depletions of this order were never feasible, and even their severest depletions could have been met, at least in theory, merely by total suppression of normal plasma protein breakdown, i.e. without calling on reserves in other pools.

It seems also that the body must possess a large and rapidly exchanging protein pool, if only to take care of sudden dietary influxes of amino acids. After a single large protein meal, amino acids may arrive in the plasma equivalent to some 50 to 100 times the amount normally present. It is clearly important that the plasma amino-acid level should not be allowed to fluctuate too widely if serious renal loss of valuable amino acids is to be avoided. The fact that smooth specific

activity curves of the type shown in Figs. 1 and 2 have been obtained at all in tracer experiments with animals on normal dietary regimes testifies to the presence of an adequate buffering mechanism. Thus, the normal amino-acid level must be determined primarily by the constants of exchange reactions with a large mass of tissue protein, and not as is often supposed by a few opposing factors such as liver uptake and renal excretion.

II. 3. *Labelling of plasma proteins*

Albumin and globulin curves in Figs. 1 and 2 have general shapes which are now known to result from single injections of various isotopically labelled amino acids into a variety of animals (cf. KINSELL *et al.*, 1950; NIKLAS and MAURER, 1952; MILLER *et al.*, 1949; VOLWILER *et al.*, 1955). Since a broadly similar general pattern results from single injections of labelled proteins it may be inferred that the effective period of production of highly labelled molecules from labelled amino acids is quite short, and that specific activities of intracellular amino acids decline rapidly at first as do those of plasma amino acids. However, values will never be entirely negligible even in the later stages of an experiment, and new molecules formed from them will have significant specific activities. The presence of such new molecules must affect the slopes of plasma-protein specific activity curves, and only after assessing the magnitude of reutilization is it possible to deduce metabolic elimination rates from experiments involving injections of labelled amino acids. Since sufficiently accurate free amino-acid measurements are not yet available in most cases, this can seldom be done. The general effect of reutilization must be to prolong protein half-lives, since free plasma amino-acid specific activities decline more slowly than do plasma-protein specific activities.

It is also not readily possible to dissociate metabolic from other effects in the period before maximum activities are reached. New labelled molecules are diluted as they arrive in the plasma with existing unlabelled ones, so that the maximum activity attained is a function both of the rate of production and of pool size. One might at first sight be inclined to assume that, if albumin and globulin have similar maximum specific activities and their plasma concentration ratio is for example 2:1, then albumin is being produced (or turned over) twice as fast as globulin. This would not be justified however because it ignores the fact that both proteins are in continuous exchange with the same proteins in the lymph, using the latter term to imply a fluid containing all the extravascular albumin and globulin of the body. That this is so was shown directly by WASSERMAN and MAYERSON (1951) and by BENSON *et al.* (1955) by injecting ^{131}I proteins into dogs and rats provided with lymphatic cannulae. Their results also showed

incidentally that plasma proteins equilibrate at very different rates with lymph in different organs and parts of the body.

After injecting a labelled protein, the plasma-protein specific activity falls steeply at first to about half the original value, showing that an amount of protein exists outside the circulation equal to that inside, and that the two are in rapid exchange. Thereafter a much slower process of final equilibration takes place in the rat (Fig. 7), suggesting the existence of a small amount of more slowly equilibrating lymph. The rapid process must greatly affect the apparent rate of appearance of new labelled molecules in the plasma when labelled amino acids are given. Even in the first hour a substantial proportion of new molecules will have escaped into the lymph, and since McKee *et al.* (1952) have shown that albumin passes across capillary membranes faster than does globulin, the effect on albumin specific activities will be greater than on globulin values. Surprisingly, this early phase of experiments with labelled amino acids seems to have escaped attention. It should be possible to measure the lymph/plasma equilibration rate independently, or even in the same animal with the help of a differently labelled protein, and to make allowance for it. When this is done the albumin maximum should be increased more than that of globulin, and apparent efficiencies of incorporation of the label into both proteins will be substantially higher than values based on the measured maximum specific activities. The slopes of rising portions of specific activity curves (assumed to be exponential, cf. insert to Fig. 1) when corrected in this way will still have a complicated significance however.

There is every reason to believe that the production of plasma proteins takes place in a regular way, i.e. there is a steady rate of net transfer of amino acids from a precursor intracellular pool into the protein pool. In this event, and neglecting the effect of metabolic breakdown in the first few hours, an exponential rate of build-up of labelled protein in the plasma should reflect an exponential rate of decline of labelled amino acid in the precursor pool. It would be interesting therefore to compare precursor amino-acid specific activity curves "reconstructed" in this way for a number of proteins with each other and with the specific activity curve of amino acids in the common plasma pool. However, additional complications enter into these considerations as will be shown in the next section in which factors governing the distribution of proteins between lymph and plasma are discussed.

III. Distribution of Labelled Plasma Proteins in Intra- and Extra-vascular Spaces

It is important in practically all *in vivo* studies of the behaviour of proteins in the plasma to know accurately the relative masses of the

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same proteins to be found outside the circulation. This is only possible by measurements following the injection of a labelled protein into the bloodstream if the time course of specific activity in every lymph compartment is known, and if it is permissible to assume that mixing is complete in the sense that specific activities are the same throughout each compartment at any one time. Since the various compartments cannot all be sampled, and indeed it is difficult to obtain a truly representative sample from even one of them, this is clearly an unattainable

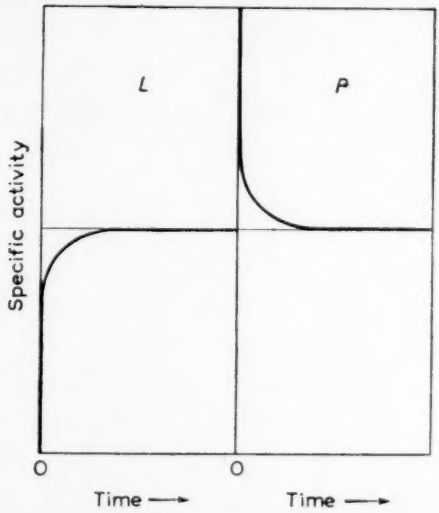

Fig. 3. Twin-compartment equilibrium system with no metabolic breakdown process. Labelled molecules injected into P.

ideal. Efforts have therefore been directed by many investigators towards extracting the maximum information from measurements made on the plasma with the full realization that a number of more or less dangerous assumptions will have to be made.

If the known multiplicity of lymph compartments (mesenteric, hepatic, muscle lymph, etc.) are visualized as a single idealized equivalent compartment (L in Fig. 3) in which all labelled protein molecules were derived exclusively from labelled molecules injected in the first instance into the plasma compartment P, then for a time net transfer of labelled molecules (or of activity) will take place from P to L, the motive force being the kinetic energy of protein molecules. This process has been called one of *interfusion* by SHEPPARD and HOUSEHOLDER (1951) to distinguish it from *diffusion* which is motivated by a concentration gradient. If no other processes operate, net transfer of activity will cease at an equilibrium time when specific activities are the same in both compartments. (SHEPPARD and HOUSEHOLDER

point out that, if there are several lymph compartments all communicating with a central one, even the relatively simple interfusion equations cannot be solved rigidly without information derived from measurements on all of them.)

If, however, labelled molecules are being selectively lost from one compartment, say P in Fig. 4, or if random breakdown and replacement with unlabelled molecules is occurring in it, specific activities in this compartment will continue to fall. They will rise at first in the other compartment (as in Fig. 3) until specific activities are the same in both

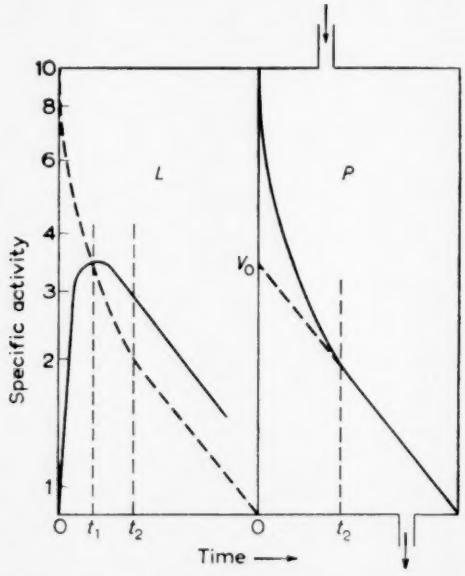

Fig. 4. Idealized lymph (L) and plasma (P) compartments with random metabolic breakdown and replacement by unlabelled molecules confined to P. t_1 = true equilibrium time and t_2 = apparent equilibrium time. Note that specific activity of lymph protein after t_2 is constantly higher than of plasma protein at the same time (cf. CAMPBELL *et al.*, 1956).

and when this is so no interfusion or transfer of activity will take place. Thereafter specific activities must be higher at all times in L than in P, and a redistribution of labelled molecules will proceed between compartments involving net transfer from L to P in an effort to make good the losses from P. Thus the time at which *maximum* specific activity is reached in the L compartment (t_1) is also the only time at which specific activities in both compartments are the same. This critical time we will call *the equilibrium time*. The relationship described here between specific activity curves of the same substance in two physical compartments is also essentially that of precursor and

product in the case of a chemical transformation of one labelled substance into another considered by ZILVERSMIT *et al.* (1943).

The equilibrium time should be distinguished from a later time (t_2) at which an *apparent* equilibrium is reached, i.e. the time at which the exponential part of the plasma specific activity curve begins. The slope of the subsequent linear semilogarithmic curve is not due, as is often assumed, solely to metabolic loss and replacement, but always includes a contribution due to continuous redistribution. This was fully realized by REINER (1953), SOLOMON (1953), WRENSHALL (1955), and BERSON and YALOW (1954), and was made the basis of generalized

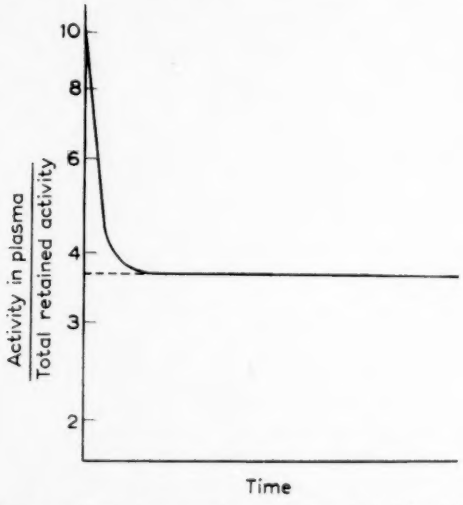

Fig. 5. If plasma activity is a constant fraction of total retained activity, this fraction equals $\frac{P}{L+P}$ (BERSON, YALOW, SCHREIBER and POST, 1953).

mathematical treatments by them in several recent papers. These authors all conclude that further progress depends on more information becoming available with which to dissociate metabolic from redistribution effects.

III. 1. *Extrapolation method*

Efforts naturally continue to be made to obtain even approximate estimates for total extravascular protein. The most popular method (STERLING, 1951) is to extrapolate the post-equilibrium part of the plasma specific activity curve to zero time, obtaining thereby a hypothetical specific activity of lymph and plasma protein (*Vo*, Fig. 4) on the assumption that the injected activity was distributed evenly in both compartments. For the reasons given, use of this method assumes that redistribution is negligible in relation to metabolic breakdown, i.e.

that specific activities in both compartments are the same. The method is sometimes applied in a different way (BERSON and YALOW, 1954) by plotting the ratio of total activity in one compartment to that in both against time, assuming the mass of protein in each to remain constant. If this ratio is found to be constant (Fig. 5) it is taken to be the mass ratio of protein in one compartment to that in the system as a whole, which clearly it will only be if specific activities in the two compartments are the same. In practice total retained activity is assumed to be equal to injected activity less activity excreted in the urine.

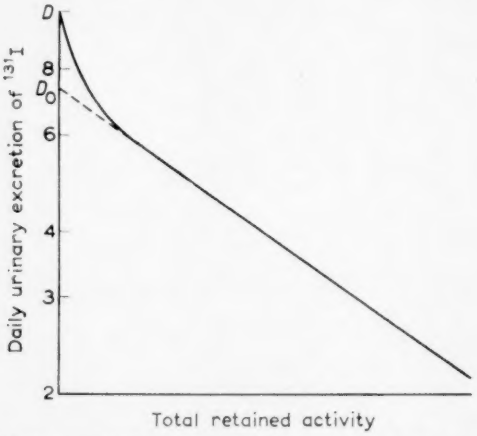

Fig. 6. If activity excreted per day in the urine settles down to become a constant fraction of total activity retained in the animal then, *D-Do* represents activity associated with altered labelled protein, and should be subtracted from the injected dose for metabolic purposes (cf. BLAHD *et al.*, 1955).

In both versions of the extrapolation method, if some of the injected activity is not present in either compartment, e.g. if it happened to be metabolically isolated in lymph ducts, or if it was excreted in addition to normal metabolic excretion, the result would be a proportionate exaggeration of the L/P ratio. A method proposed by BLAHD *et al.* (1955) to circumvent this difficulty is to plot urinary excretion against total activity in the animal. Assuming metabolism to proceed in parallel with total body activity, this should give a straight line when plotted on a linear scale. In practice urinary excretion was found to lie above the extrapolation line at first (Fig. 6), and this was attributed to the presence of altered labelled protein which the animal had rejected and broken down in the first few days. The authors conclude that superfluous activity represented by *D-Do* should be subtracted from the injected dose to give the effective dose for subsequent metabolic processes. For a group of normal humans *D-Do* was found to be equivalent to approximately 10 per cent of the injected dose. The

method rests on the unlikely assumption that metabolic breakdown occurs in both compartments in proportion to the mass of protein in each. Thus both extrapolation methods have an insecure theoretical basis and also have the practical disadvantage that they depend on critical measurements of specific activity made on plasma samples which must be taken after the injected molecules have fully mixed in the plasma and before any significant loss from the plasma has had time to occur.

III. 2. *Equilibrium time method*

Evidence has been presented by CAMPBELL *et al.* (1956b) in favour of the view that albumin and γ globulin are mainly if not exclusively metabolized in rats in sites which are closely associated with the plasma and correspondingly removed from the lymph. The evidence depends on a finding of parallelism between rate of urinary ^{131}I excretion and of total plasma protein activity, and is illustrated by plotting so-called *excretion (or "metabolic") rates*, i.e. 24 hr urinary ^{131}I activities expressed as functions of mean prevailing plasma protein activities over the corresponding period (Fig. 7). During the first few days when the total activity associated with protein in the plasma was falling rapidly, the excretion rate as defined above was constant. On the other hand, if urinary activity was expressed as a function of *total* activity in the animal as a whole rather than in the plasma, the excretion rate fell rapidly at first.

Comparison of the behaviour of the same ^{131}I with similar ^{14}C-labelled proteins (Fig. 8) showed no evidence for an exceptional loss of ^{131}I proteins from the plasma in the first few days. Corroborative evidence comes from studies by BERSON and YALOW (1954) of human nephrosis in which urinary ^{131}I activities followed plasma-protein specific activities closely when ^{131}I albumin was injected either into the circulation or into the peritoneum, although in the first few days the plasma protein specific activity curve followed a rising course in one case and a declining course in the other. BAUER *et al.* (1954) also observed similar effects in their study of cirrhotic patients with ^{131}I albumin, and concluded that catabolic turnover must be occurring primarily in the vascular compartment. HUMPHREY and MCFARLANE (1955), showed after injecting [^{14}C] amino acids, that albumin and normal γ globulin reached maximum specific activity in the plasma of rabbits in 4 to 5 hr, whereas antibody globulin produced in an extravascular site took 50 or more hours to reach maximum concentration in the plasma. Furthermore, since MILLER and BALE (1954) showed that albumin is exclusively produced in the liver, and hepatic rat lymph proteins are in rapid exchange with plasma proteins (BENSON, KIM and BOLLMAN, 1955), the case for a functionally vascular origin for this protein at least appears to be made. In what follows it is also

necessary to assume that breakdown as well as replacement occurs in the plasma compartment, and evidence which will be discussed in due course is available, indicating that the liver may also be an important site of protein catabolism.

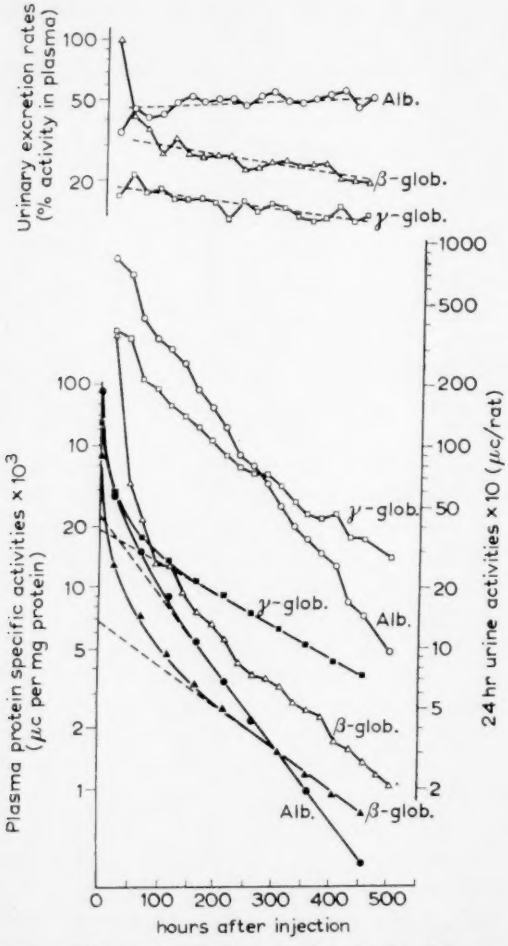

Fig. 7. Plasma protein ^{131}I specific activities (in blocked symbols) after injecting individual labelled proteins into groups of normal rats. Also shown are urinary 24-hr-^{131}I excretions (in open symbols) and the same values (top of figure) plotted as functions of mean total activities in plasma during period of excretion (so-called "excretion" or "metabolic rates") (from CAMPBELL *et al.*, 1956).

First we may assume, as is frequently done, that for an adult animal in protein equilibrium all protein broken down is more or less promptly replaced, and in addition we will make the less justifiable assumption that metabolic breakdown and replacement can be represented by a

single equivalent balanced rate process which we will call *the metabolic process*. (If breakdown and replacement occur in different compartments the following considerations will not necessarily apply.) Considering the hypothetical 2-compartment system in Fig. 4, it has been shown mathematically by CAMPBELL *et al.* (1956b) that an experimental finding of linearity in the semilog plot of specific activities in one compartment after apparent equilibrium has been established justifies

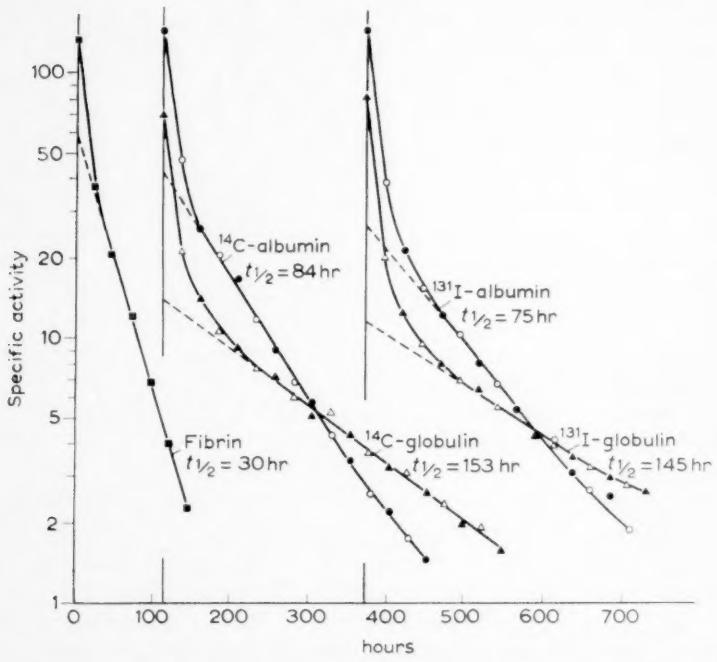

Fig. 8. Comparison of elimination of ^{14}C- and ^{131}I-labelled rat proteins injected into comparable groups of normal rats (from CAMPBELL *et al.*, 1956b).

certain approximations, which in turn imply that specific activities in the other compartment must also be linear over the same period. Also, whereas extrapolation methods of measuring L/P ratios depend on the critical assumption of *identical* specific activities in both compartments, the following conclusions only require that they shall have a constant ratio. Only at the critical time t_1 are specific activities the same in both compartments. At this time *total* activity in the extravascular compartment reaches a maximum, since for a constant mass of protein in the compartment the time curves of total and specific must be parallel. The product of plasma specific activity and of mass of protein in the circulation gives the total activity in the plasma.

The ratio of total *activities* in the two compartments at equilibrium is also the ratio of the protein *masses* in each (or the L/P value) since at this time specific activities are the same in both compartments.

A distinctive feature of this method of deriving L/P ratios is that it does not depend on the specific activity of an early plasma sample. Accurate measurements which are required to find the equilibrium time are of proximal plasma protein specific activities and urinary ^{131}I activities, and of total ^{131}I excreted. Thus the experiment need not last more than 2 or 3 days. The use of urinary information to determine retained activity suggested by BAUMAN *et al.* (1955) assumes that excretion is exclusiyely renal. CAMPBELL *et al.* (1956b) illustrate the use of an alternative method based on total body γ-radiation measurements.

Faecal excretion in the rat in 24 hr is 2 to 5 per cent of the urinary activity in the same period, but accurate information on this point with different labelled proteins in different species and over the full period of an isotope experiment is not available. Salivary excretion may also not be ignored. Urinary excretions increased by 10 per cent may be taken to represent total excretions fairly closely. The injected dose must be known accurately and may be checked from the measurement of specific activity of the first plasma sample if the mass of protein in the circulation is known.

Approximate values derived for L/P in the rat by this method both with albumin and globulin (CAMPBELL *et al.*, 1956b) were 1·3 to 1·6. These values were derived from measurements on an inadequate number of blood and urine samples available in the neighbourhood of the equilibrium point, and are to be regarded as provisional. If any significant proportion of injected labelled molecules had been prematurely metabolized, the activity thus liberated from protein combination and appearing in the urine (MCFARLANE, 1956) would not have affected the estimations of retained dose or the L/P values. This would not be true however if labelled molecules were retained in the animal in such a way that they had ceased to exchange in any physicochemical way with similar molecules in lymph and plasma.

III. 3. *Activity distribution method*

Using the same basic model, i.e. a 2-compartment equilibrium system with breakdown and replacement confined to one compartment, CAMPBELL *et al.* (1956b) have shown that the ratio of activities at any time in the two compartments is given by the ratio (after subtracting unity) of metabolic rate (or urinary excretion rate, cf. Fig. 7) to plasma protein apparent replacement rate, the latter being obtained from the slope of the plasma protein specific activity-time curve. This activity ratio can be used to obtain L/P values if specific activities in both compartments are known, which is

generally not the case. However, if lymph specific activities are assumed always to be higher than plasma values at the same time, the *activity distribution ratio* gives a valuable measure of the maximum L/P value. The ratio in this case also can be measured at any time after equilibrium is achieved, and therefore depends only on the behaviour of labelled molecules which have survived in the lymph and plasma after the first few days, i.e. it is independent of the presence of denatured material in the labelled protein which was injected.

In the rat the activity-distribution method gave practically the same maximum value for L/P whether measured with ^{131}I albumin or ^{131}I-γ globulin. Values were in the same range (1·3 to 1·6) as those arrived at for L/P by the equilibrium time method, implying that differences in specific activities of proteins in lymph and plasma compartments in this animal must be small. On the other hand, values measured in the same animals by the extrapolation method were considerably higher (2·2 to 3·0), indicating an initial extrametabolic loss of activity. The observation that an equally large apparent loss occurred after injecting ^{14}C-labelled proteins suggests that it may be part of a physiological phenomenon such as temporary passage of lymph and plasma proteins into a compartment in which they cease for practical purposes to be metabolized or redistributed. Lymph in lymphatic ducts could theoretically comply with the requirements, but it is not necessary to give the compartment an anatomical definition. The observed phenomena could also be explained by non-ideal mixing in several or all of the real lymph compartments combined with slow return flow of lymph to the plasma via lymphatic ducts.

Metabolic isolation of a lymph fraction alone is inadequate to explain the facts, and in any case labelled molecules would not penetrate into an isolated compartment in the first instance. The essential point is that a constant proportion of lymph and plasma protein should always be in transit. In the isotope experiment this protein will be constantly returning to the plasma with a specific activity which is higher than that of plasma protein at the same time. From what is known of the physiology of the lymph system a specific activity discontinuity could occur at the junction of lymph and plasma at the thoracic and other lymph-returning ducts, but the possibility is one which is extremely difficult to examine mathematically or experimentally.

A mechanism of this kind which in effect virtually syphons incompletely mixed lymph back into the plasma with a time-lag should have the following consequences. It should increase the total activity in the plasma and give rise to L/P values by the *equilibrium time* and by the *activity-distribution* methods which are too low. It should give rise to apparent half-lives of plasma proteins longer than the true

metabolic half-lives and to L/P values by the extrapolation method which are too high. Since an increasing proportion of all labelled molecules in the body would become effective in a metabolic sense after the first few days, the ^{131}I-excretion rate should increase during the tracer experiment as indeed has been observed in rats receiving ^{131}I-albumin (CAMPBELL *et al.*, 1956b). However, the value of this observation is much reduced by the fact that the opposite was found to be true of rats receiving ^{131}I-γ globulin.

IV. SOME PRACTICAL CONSIDERATIONS GOVERNING THE PREPARATION AND USE OF LABELLED PROTEINS

In general, plasma proteins which have been labelled in the animal, or so-called biosynthetically-labelled proteins, are to be preferred to proteins labelled *in vitro*. However, the possibility of adversely affecting the properties of the former in the course of transference from donor to recipient must be kept in mind. Direct transference is seldom permissible because labelled plasma is usually harvested when the plasma proteins are maximally labelled, and at this time significant activity is associated with amino acids and other diffusible substances. These should be removed by dialysis against changes of carrier substances as described by DOVEY *et al.* (1954). Also when the objective is to study the separate behaviours of labelled proteins it may be necessary to fractionate the plasma or perhaps to freeze and dry it, and the extent to which popular procedures for doing these may affect turnover rates is not known.

Following the experiment of MILLER *et al.* (1949) with [ε-^{14}C] lysine, the use of specifically labelled amino acids to label donor animals has declined probably on grounds of expense, except perhaps in the case of [^{35}S] cysteine or methionine. These have been used by KINSELL *et al.* (1950) in humans, by MADDEN and GOULD (1952) in dogs, and by NIKLAS and MAURER (1952) and by LEE and WILLIAMS (1952) in rats. Yeast grows well on a medium containing [^{35}S] sulphate, and the yeast protein may be fed to animals or hydrolysed as a preliminary to chromatographic separation of [^{35}S] cysteine (cf. ARMSTRONG *et al.*, 1954b). A valuable source of [^{14}C] amino acids is to be found in dried ^{14}C *Chlorella* or the protein extracted from it by CATCH (1954) with specific activities of 100 microcuries per milligramme. Since the alga is grown on $^{14}CO_2$ as sole source of carbon, it offers the important advantages that all carbon atoms are labelled and of course all the amino acids are in the biologically useful L forms. PIHA (1954) has shown that it is particularly useful for labelling erythrocytes. In common with all biosynthetic labelling procedures the use of this protein is wasteful, since only a few per cent of the activity given is recoverable in the form of labelled plasma proteins.

IV. 1. *Labelling with* ^{131}I

Apart from a few preliminary experiments by GRAY and STERLING (1950) with isotopic chromate, the much more economical *in vitro* labelling of plasma proteins has been almost exclusively concerned with the use of iodine. The fact that this element combines so readily with plasma proteins has possibly not been altogether beneficial. The tendency for some years was simply to add isotopic iodine kept in solution with the help of iodide to neutral or alkaline protein solutions, the yellow iodine colour disappearing immediately and up to half the isotope becoming nondiffusible through cellophane. By all animal tests the isotope did not become separated again from the protein short of metabolic disintegration of the latter, and subsequently the label was not significantly reused or retained in the animal provided the thyroid had been blocked beforehand with iodide.

A great deal of valuable work was done with this material by GITLIN *et al.* (1951), by FRANCIS *et al.* (1951), by TALMAGE *et al.* (1951), and by MELCHER and MASOUREDIS (1951), STERLING (1951) and others. However, following reports of variable results, investigators tended to use progressively less iodine to a given weight of protein. GABRIELLI, GOULIAN, KINERSLEY and COLLET (1954) noted at least two fractions by zone paper electrophoresis of commercially available ^{131}I-human serum albumin. BERSON *et al.* (1953) showed that different batches of similar material gave different rates of urinary ^{131}I elimination, and evidence of a similar nature came from VAUGHAN *et al.* (1955). VOLWILER *et al.* (1955), and also ARMSTRONG *et al.* (1954), concluded that iodinated plasma proteins were more rapidly metabolized than their ^{35}S counterparts, and MASOUREDIS and BEECKMANS (1935) found ^{131}I-labelled proteins to compare unfavourably with ^{14}C-labelled ones.

COHEN *et al.* (1956) compared the behaviour of ^{14}C-labelled rabbit albumin and globulin in the same rabbit with albumin and globulin which had been iodinated to the extent of 1 atom iodine per molecule, using a mechanical arrangement to distribute the label evenly throughout the population of molecules. They found close agreement between the two (Fig. 9), while MCFARLANE (1956) found poor agreement when the proportion of iodine was increased to 6 atoms per molecule. Comparisons were based on measurements of apparent replacement rates and of lymph/plasma distribution ratios using the extrapolation method of STERLING (1951). Although the precise significance of the latter ratio is not clear for reasons already given, it offers a more useful basis of comparison than replacement rates alone, since it is possible to iodinate a protein in such a way that a large proportion is promptly rejected by the recipient animal, but the part which survives is

metabolized at a normal rate. Prompt rejection of part of the ^{131}I protein was revealed by an unusually high L/P ratio combined with a high rate of urinary ^{131}I excretion in the first day or so. The reliability of an iodination procedure was also tested by McFarlane (1956) by transfusing plasma from a recipient animal 2 or 3 days after the injection of labelled protein to a similar animal, and comparing L/P ratios in the two. Iodinated rat albumin and globulin also behaved in the same way as corresponding ^{14}C-labelled proteins (Fig. 8), the comparison in this case being made in similar groups of animals.

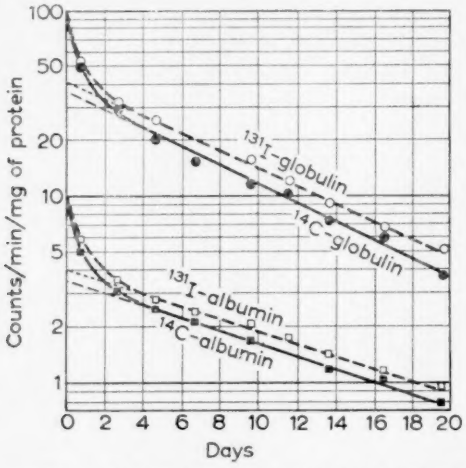

Fig. 9. Comparison of elimination of ^{14}C- and ^{131}I-labelled rabbit proteins injected into the same rabbit (from Cohen *et al.*, 1956).

As is now generally recognized, a satisfactory iodinated protein should show no difference in behaviour in the ultracentrifuge from the uniodinated protein, and it should contain less than 2 per cent of diffusible ^{131}I activity. The use of an ion-exchange resin to remove iodide from the iodination mixture appears to be a safe and efficient procedure (Sterling, 1951; Anigstein *et al.*, 1954) although in the case of iodinated rabbit plasma some protein was retained by the resin (McFarlane, 1955). Proteins which had been in contact with methanol in the cold, or human albumin which had been heated at 55°C, did not iodinate satisfactorily, suggesting that any strain on the molecule additional to that arising from accommodating the relatively large iodine atom in the tyrosine ring is likely to lead to preferential treatment by recipient animals.

IV. 2. *Growth and sample corrections*

It is highly desirable in studying the behaviour of labelled plasma proteins in normal animals that these should be in protein equilibrium

over the often lengthy periods of tracer observations. This is seldom achieved in practice, since most laboratory animals are growing and plasma proteins are often being removed in the form of blood samples. If only to make results comparable with those of others measured, values should be corrected to give values which would have been obtained if the measurements had been made on animals in nutritional equilibrium. For example, a 250-g rat increases its body weight and correspondingly its lymph and plasma proteins by about 1 per cent per day. The new protein is usually poorly labelled or unlabelled, and hence it lowers the specific activity of lymph and plasma proteins almost proportionately. Thus the measured apparent replacement rate of rat γ globulin, which is 9·5 per cent per day (Fig. 7), would have been 8·5 per cent per day if it had been measured in the absence of growth.

A plasma sample of 0·22 ml taken from such a rat represents approximately 1 per cent of the lymph and plasma protein pools. If plasma samples are taken daily this correction also will amount to 1 per cent per day to be subtracted from the measured replacement rate. In very young animals or in older animals recovering from plasma depletion the corrections will be even larger. Also, for reasons already given, if new lymph and plasma proteins are discharged first into the vascular pool, the corrections which must be applied to specific activities measured in the vascular compartment may require to be substantially increased and in an unpredictable way. In general therefore it is desirable to reduce corrections as far as possible by minimizing blood sampling and by using adult animals. Where blood sampling has been carried out at irregular intervals, or where different volumes of blood have been removed, the effect of failure to apply the proper corrections may be to obscure the exponential nature of specific activity curves. Examples of this are to be found in papers by Dovey *et al.* (1954) and by Volwiler *et al.* (1955).

V. Metabolic Behaviour of Transfused Labelled Proteins

Possibly the most important prerequisite for an understanding of the behaviour of labelled protein molecules is an assurance that there has been no dissociation of the label in lymph or plasma. The demonstration by Heidelberger *et al.* (1942) that pneumococcus antibody passively transfused to rabbits receiving [^{15}N] glycine in the diet did not take up the isotope, makes quite clear the fact that this amino acid at least does not exchange with the same amino acid in fully formed antibody globulin. A similar result was obtained by Gros *et al.* (1952) using labelled valine. Humphrey and McFarlane (1954a) transfused pneumococcus type III antibody biosynthetically labelled with ^{14}C

in all carbons to rabbits along with similarly labelled normal γ globulin and followed antibody concentrations and globulin specific activities in the plasma for three weeks (Fig. 10). These declined at almost the same rate—12·5 per cent per day—and at the end of this time when only a few per cent of the transfused antibody had survived in the circulation some of it was isolated by specific precipitation and found to have precisely the same specific activity as the injected material. This shows that antibody globulin and possibly all plasma protein

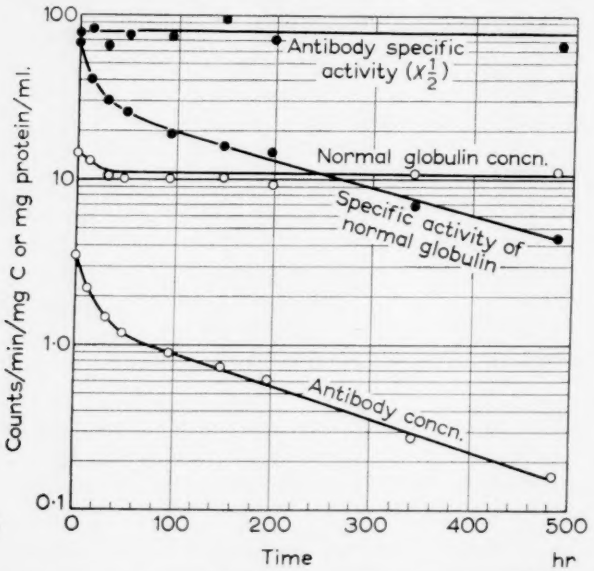

Fig. 10. Behaviour of rabbit ^{14}C-labelled pneumococcus antibody and normal γ globulin transfused to normal rabbit (HUMPHREY and MCFARLANE, 1954a).

molecules can circulate in lymph and plasma for a period of three weeks without significantly exchanging any of their constituent amino acids.

It is unfortunate that the essential slowness of lymph/plasma equilibration as it effects injected labelled molecules was not at first fully realized, and experiments were frequently not carried on long enough for truly exponential portions of specific-activity curves to become fully evident. Thus in the first important instance of the use of labelled proteins in this way—by MILLER *et al.* (1949) in dogs—measurements terminated at 6 days, whereas apparent equilibrium in humans, rabbits and rats is not established until about this time. For this reason there is scarcely enough information available to make it profitable to discuss relative replacement rates for different proteins in different species as one would like to do. In addition, practically nothing is known of the effects, if any, of diet, age, sex and exercise on

replacement rates, although STEINBOCK and TARVER (1954) have shown that rates are greatly affected by the level of protein in the diet of rats.

The most that can be said with any assurance is that broadly speaking the smaller the animal the more rapidly it breaks down and replaces its lymph and plasma proteins. Half-lives as short as 1 to 2 days have been obtained by MELCHER *et al.* (1953) in mice, while the half-life of albumin and globulin in humans lies between 8 and 25 days (KINSELL *et al.*, 1950; LONDON, 1950; STERLING, 1951; MYANT, 1952; EISENMENGER and SLATER, 1953; HAVENS *et al.*, 1953; ARMSTRONG *et al.*, 1954a; BAUMAN *et al.*, 1955; VAUGHAN *et al.*, 1955).

Fibrinogen appears to be turned over faster than other plasma proteins (MADDEN and GOULD, 1952; DOVEY *et al.*, 1954; ARMSTRONG *et al.*, 1955; COHEN *et al.*, 1956). As a rule the rate for albumin is slower than for γ globulin. However, CAMPBELL *et al.* (1956b) found in the rat (Fig. 7) that albumin at 20 per cent per day was replaced more than twice as fast as γ globulin, the rate for fibrinogen being no less than 60 per cent per day. Very little is known about the rates for α and β globulins, although LONDON (1950) is of opinion that they are replaced in humans faster than albumin or γ globulin. The curve in Fig. 7 suggested a value for β globulin in the rat which was intermediate between those of albumin and γ globulin, but the rapid excretion of label in the urine indicated that the β globulin used in this experiment was far from homogeneous either before or following the iodination. From measurements made after 4 to 6 hr using [^{14}C] lysine, MILLER and BALE (1954) concluded that α globulin is turned over faster in the rat than any other plasma protein. However this is based on finding a high *total* ^{14}C activity in this fraction, and it is hardly permissible to attribute it all to protein. Various substances in plasma other than proteins become highly labelled at this time, and some of them may migrate with α globulin. For example BOAS (1955) showed that rat α globulin contains 4·4 per cent glucosamine.

The behaviour of ^{14}C- and ^{131}I-labelled proteins in rats may be contrasted with their behaviour in rabbits (Figs. 8 and 9), and with the behaviour of serologically labelled globulin in the same animal (Fig. 10). The serological label has much to recommend it. HUMPHREY and MCFARLANE (1954a) noted a slightly faster apparent elimination of antibody globulin than of ^{14}C-labelled normal γ globulin, and concluded that this was due to some reutilization of the isotopic label. They also observed that lymph/plasma equilibration of antibody was usually complete at 25 hr, suggesting that the slower equilibrations observed with labelled autogenous γ globulin may be due to extravascular synthesis of a portion of normal rabbit globulin combined with slow transference of the new protein to the plasma via the lymphatic

system. In view of complications which may conceivably arise in turnover studies if synthesis and breakdown of protein occur in different compartments, it is clearly an important advantage of the serological label that the results obtained with it are independent of synthesis. On the other hand, to infer turnover rates of autogenous globulin from the elimination rate of antibody requires an assurance that the latter cannot be distinguished by other than serological means from the non-immune globulin, and furthermore that recipient animals are reliably free of antigen to the particular antibody used.

Dovey *et al.* (1954) discuss the remarkable fact that albumin, γ globulin and pneumococcus antibody were all turned over or eliminated at approximately the same exponential rates in rabbits. This was true whether antibody was present initially at a plasma level of 35 mg/ml or at only one-tenth this level, and the authors suggested therefore that, if an exception can be made in the case of fibrinogen, the elimination of plasma proteins may take place by phagocytic mass removal of whole plasma or lymph. The view would still be tenable even if there were small differences in apparent plasma-elimination rates in view of redistribution effects which are inherent in them and which have already been discussed. However, the information derived subsequently from rats appears to challenge the proposal seriously. It is hardly possible to visualize a lymph/plasma redistribution effect on rat γ globulin of such magnitude that the true elimination rate of this protein at the site of elimination could be the same as that of albumin in the same animal. The evidence in this case suggests on the contrary that albumin, globulin and fibrinogen are eliminated by independent metabolic processes.

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VI. The Isolated Perfused Liver

A notable contribution to the whole field of plasma protein physiology was made by Miller and his associates using the freshly isolated perfused rat liver. Miller *et al.* (1951) continuously circulated oxygenated blood through the organ for 9 hr or more in a constant-temperature chamber, during which time bile was secreted at satisfactory rates, CO_2 and urea were produced, glycogen was stored, and plasma proteins formed. Six hours after introducing *DL*-[^{14}C] lysine into the circulating plasma, some 20 per cent of the activity had appeared as CO_2, clottable labelled fibrinogen was present, and up to 10 per cent of the activity was associated with plasma proteins depending on the level of amino acids in the plasma. Utilization of *L*-lysine for these purposes was apparently almost complete whereas the unnatural *D* form was totally unused. Production of labelled plasma proteins gradually declined and practically ceased at 4 hr, but resumed at once at the former rate when fresh amino acids were introduced.

The authors conclude "The final flat or declining phase of the ^{14}C-incorporation into plasma proteins is not a result of weakening, exhaustion, or failure of the surviving livers, but is primarily related to the decrease and disappearance of essential substrates."

According to Fig. 11 the amount of labelled plasma and liver proteins produced after 6 hr is almost proportionately related to the weight of

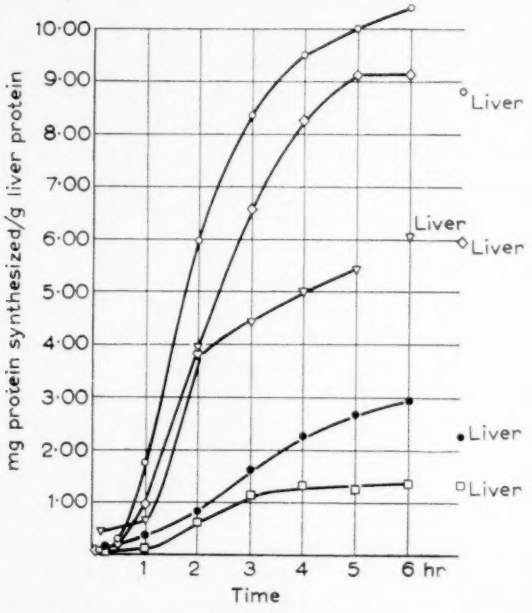

Fig. 11. From MILLER *et al.* (1951).

total amino acids given in the form of a balanced supplement up to approximately 330 mg. The weight of plasma protein synthesized with the help of this supplement (ca. 20 mg by a liver containing 2 g protein) is claimed by MILLER *et al.* (1954) to be sensibly the same as that synthesized in the same time in the intact rat. The amount synthesized by the perfused organ when 1·74 mg *DL*-[^{14}C] lysine was given without any supplement was apparently less by a factor of 50 despite the fact that 100 ml fresh rat plasma was used containing presumably about 50 mg amino acids. This plasma came from rats which had been fasted for 18 hr, and the authors imply that its amino acids were not adequate either in quantity or quality to support plasma-protein

formation at the normal rate. They also calculate, on the basis of 20 mg plasma protein produced in 6 hr from 327 mg amino acids, that the intact liver of a 300-g rat is fully able to convert *all* dietary amino acids from a 20 per cent protein diet continuously into plasma proteins. They point out that it is also favourably placed to do so by virtue of its situation on the portal vein.

Before accepting these conclusions, which have far-reaching implications, it is necessary to examine more closely the form of calculation by which radioactivities were converted into weights of plasma or liver proteins. The authors say "Thus, in a typical experiment, if 20 mg of *L*-lysine were present in the amino-acid mixture added, and had an arbitrary activity of 250 units, and if 2·0 g liver protein were found to contain 4·0 units of radioactivity per gramme, then $2(4/250) \times 20 \times 100/7 = 9{\cdot}2$ mg of liver protein made by the entire liver (2 g on 'dry' protein basis)." The lysine content of plasma and liver protein is taken to be 7 per cent.

In effect this form of calculation assumes that plasma proteins are made directly from plasma amino acids which have constant specific activity and this is certainly not true. The specific activity curve of plasma-free amino acids is in fact falling (as the authors show, to about 15 per cent of the initial value in 6 hr after giving *L*-[^{14}C] lysine), and the mean value effective in plasma-protein synthesis over a period of 6 hr must be substantially less than the initial value. In addition, intracellular amino acids which are immediate precursors of plasma proteins almost certainly have a still lower mean value. This arises from the fact that part of the protein of the initially unlabelled liver is metabolically mobile, and both contributes and receives amino acids from the intracellular pool. Twenty-five per cent (or 500 mg dry protein) is probably not an overestimate for this category of protein in the liver of a 300-g rat. Thus, we may visualize the grossly simplified perfusion system to consist, as in Fig. 12, of a centrally placed intracellular amino-acid compartment containing some 50 mg amino acids from which amino acids are continuously withdrawn to form new plasma proteins. This communicates reversibly with a plasma-free amino acid compartment containing a similar amount of amino acids plus any supplement, and with a second compartment containing amino acids equivalent to 500 mg mobile liver protein.

Evidence for a general arrangement of this kind was indeed obtained by Miller *et al.* (1955) when they perfused the liver from a [^{14}C] lysine-labelled rat without supplementary amino acids, and found 6·3 per cent of the initial liver activity in plasma proteins after 6 hr. Applying the same kind of calculation to this result as was applied by Miller, Burke and Haft to the results of their perfusion using

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labelled amino acids, the mass of plasma protein produced in 6 hr is estimated at 33 mg (6·3 per cent of 500 mg). It might have been greater, depending on the proportion of exchangeable protein in the liver, but could not have been more than 130 mg or 6·3 per cent of all the lysine in protein and in amino acids in the whole system. When an unlabelled liver was perfused with a tracer dose of labelled amino acids, only 0·3–0·5 per cent of the activity, equivalent to less than a milligramme of plasma protein, appeared in plasma proteins in 6 hr. Thus we have two entirely different estimates for plasma protein pro-

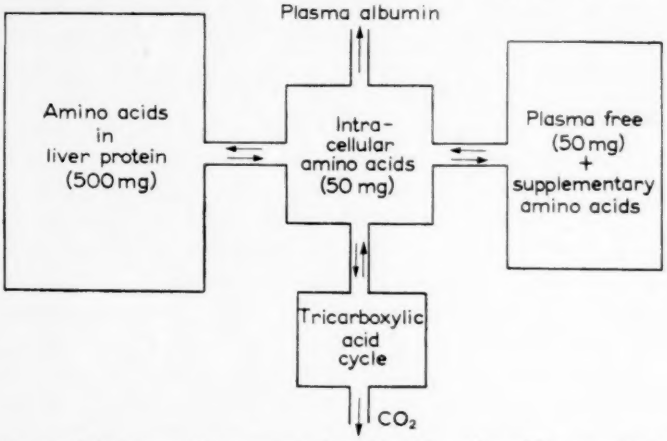
Plasma albumin
Amino acids in liver protein (500 mg)
Intra-cellular amino acids (50 mg)
Plasma free (50 mg) + supplementary amino acids
Tricarboxylic acid cycle
CO_2

Fig. 12. Diagrammatic representation of amino-acid equilibrium system in perfused rat liver.

duced by the same system and derived by essentially the same method of calculation. It is evident that each half of a dynamic system of this kind cannot be considered without reference to the other.

To convert activities to milligrammes protein requires information about the specific activity of the *immediate* precursor amino acids, and this at any time is a complicated function of specific activities of amino acids which are free in the plasma and those which are protein-bound in liver cells. If exchange rates between compartments in Fig. 12 are rapid, as there is every reason to believe them to be, the lateral pools may be expected to affect amino-acid specific activities in the central one in proportion to the mass of amino acids which they contain. This is another way of saying that equilibrium is quickly attained, and subsequently specific activities are the same in all compartments. On this basis the writer is of opinion that Fig. 11 does not necessarily therefore indicate that the liver was making plasma proteins in proportion to the weight of amino acids in the supplement, but possibly only that the effective specific activity of precursor lysine was increasing in parallel with the amount of lysine in the plasma.

The remarkable finding (Fig. 11) that total activities in liver and plasma protein after 6 hr were roughly equal whatever the amount of amino-acid supplement used is difficult to explain. When no supplement was used one would expect to find that most of the *labelled* amino acid had been transferred in a few hours from the plasma to the liver by an exchange process without reduction in the plasma amino-acid concentration. A constant or slightly increasing concentration of free amino acids in the plasma was actually observed. When supplementary amino acids were used, on the other hand, the system was unbalanced in a physiological sense and the concentration of amino acids in the plasma fell steadily, less than 20 per cent of the amino acids which disappeared from the plasma in 2 to 3 hours being converted to plasma proteins.

The value of 20 mg plasma proteins synthesized in 4 to 6 hr by a 300-g rat, or 100 mg per day, obtained by MILLER *et al.* (1954) may be compared with that of NIKLAS and MAURER (1952). These authors using [^{35}S] methionine obtained by hydrolysis of ^{35}S-labelled protein of *Torula utilis*, found that the liver of the intact rat produced plasma proteins equivalent to one-seventh of its protein weight per day. This would amount to 250 to 300 mg per day in a 300-g rat. ABDOU and TARVER (1952) found rather more rapid replacement after giving ^{14}C-labelled rat total plasma proteins to 210-g rats. CAMPBELL *et al.* (1956b) found apparent replacement of ^{131}I-labelled rat albumin and γ-globulin (Fig. 7) to take place at 21 per cent and 8·5 per cent per day respectively. Using 15 per cent per day as a rough estimate for the mean replacement rate of total proteins (amounting to 1·25 g excluding γ globulin) in lymph and plasma of a 300-g rat, then about 200 mg new protein was synthesized by the liver per day. It can hardly have been less since albumin production alone was almost equal to this value.

In view of differences which may yet be revealed in the behaviour of normal animals in different circumstances, it is probably unjustifiable to rely on a particular value at this stage, but it would appear that the liver of the normal 300-g rat cannot be making less than 150 mg plasma protein per day, and may be making up to twice this amount. Therefore the perfused ^{14}C-labelled liver in MILLER, BURKE and HAFT's experiment which we have calculated to be producing 33 mg in 4 to 6 hr without the help of supplementary amino acids may have been functioning quite as efficiently as in the intact rat, although the authors themselves regarded it as giving a poor performance.

MILLER, BURKE and HAFT also perfused an unlabelled rat liver with ^{14}C-labelled plasma proteins without supplementary amino acids; 4·5 per cent of the activity disappeared from the plasma in 9 hr, 2·7 per cent into the liver and 1·5 per cent into expired CO_2. This would

appear to show that the liver must be an important organ for catabolism as well as synthesis of plasma proteins, since 4·5 per cent of the total plasma protein in the system (4 g) is equal to 180 mg in 9 hr or 480 mg per day. Therefore this liver was capable of hydrolysing at least as much plasma protein as is regularly made by the rat.

The amount of plasma protein catabolized to CO_2 in this experiment was equivalent to 26 mg urea nitrogen per day. In the experiment already referred to in which a labelled liver was used on the other hand, *liver* protein broken down to CO_2 was equivalent to 42 mg urea nitrogen per day. The two together are approximately equal to the total urea nitrogen excreted daily by an equivalent intact rat. It will be noticed that approximately one-third of liver and plasma protein regularly broken down and replaced by the rat on a protein-free diet is deaminated in each case.

When a 300 mg amino-acid supplement was used in the typical perfusion experiment total urea nitrogen production was slightly more than doubled, the additional urea nitrogen being not quite equivalent to amino-acid nitrogen which was observed to disappear from the plasma. MILLER, BURKE and HAFT conclude that a small positive nitrogen balance was achieved, and proceed to consider the sparing effect of added carbohydrate on this balance. However, the argument rests on the assumption that the weights of liver and plasma proteins deaminated in the presence of the supplement were the same as the weights deaminated in experiments with ^{14}C-labelled liver and plasma proteins when no amino-acid supplement was given. The assumption is based on the concept of an endogenous metabolism proceeding at a basal rate which is unaffected by the arrival of amino acids in plenty, and there is no sound evidence for this. On the contrary all the evidence is in favour of a dynamic interlocking of biochemical processes so that the outcome of any change in conditions can only be predicted by reference to its effects on the system as a whole.

VI. 1. *Sites of plasma-protein synthesis*

The experiments described, using the perfused rat liver, confirmed the already widely held view that this organ is the major source of albumin in the animal body. That it is the only source was demonstrated by MILLER, BLY and BALE (1954) when they adapted their perfusion apparatus for experiments with the caudal half of the rat carcass. Their preparations comprised muscle, skeleton, and skin below the midlumber region in all cases and in a number the kidneys, pelvic viscera, gastro-intestinal tract, spleen, and pancreas, supported by the diaphragm and clamped abdominal wall. Half an hour was required between incising the rat and setting up the preparation with a warm oxygenated whole-blood supply, and the carcass was without a blood

supply for not longer than 8 min. Viability as judged by blood flow, oxygen and glucose consumption, CO_2 production, and uptake of labelled amino acids, was maintained for a maximum period of 4 to 5 hr during which time urine flowed satisfactorily.

As in the case of the perfused liver, $D[^{14}C]$ lysine was not significantly used by the carcass. With $L[^{14}C]$ lysine no activity appeared in albumin or fibrinogen irrespective of whether the gastrointestinal tract, pancreas, spleen or kidneys were included in the preparation. However, protein-bound activity was present in the plasma after 6 hr, all of it in the globulin fraction obtained by precipitation with sodium sulphate. By zone electrophoresis of the plasma it was shown that nearly all this activity was present in γ globulin, the remaining traces being confined to α and β globulins.

This result led to re-examination of an earlier conclusion by MILLER *et al.* (1951) that the liver produces 80 per cent of all globulin in the rat. This was based on the radioactivity found in globulin precipitated with sodium sulphate from perfused liver blood after 6 hr, a material which must have been a mixture of all three globulins, and may even have contained some albumin since it represented more than half the total weight of protein in the plasma. MILLER and BALE (1954) repeated the earlier work using zone electrophoresis, and showed that γ globulin was totally inactive. This important finding is compatible with the high plasma globulin levels often found in liver disease, and with the observation by BATTY and WARRACK (1955) that the liver seldom if ever produced antibodies. JACOBSON *et al.* (1950) also observed that massive doses of whole-body radiation ($L.D._{50}$ to $L.D._{100}$) given to rats and rabbits destroyed the ability to produce antibodies or normal γ globulin. This was confirmed by MILLER and BALE (1954), who also showed that similar doses given to the perfused rat liver did not depress the production of labelled plasma proteins. All the evidence therefore points to an extrahepatic origin for γ globulin, most probable sites being bone marrow and spleen (ASKONAS and HUMPHREY, 1955). α and β globulins on the other hand appear to be mainly produced in the liver.

MAURER and MÜLLER (1955) revive a proposal which has appeared many times in the literature (cf. WUHRMANN and WUNDERLY, 1953) to explain apparently compensatory changes in plasma levels of albumin and globulin suggesting that these proteins are to some extent mutually interconvertible. They claim that albumin is a precursor of α, β and γ globulins in normal rats, and suggest that this may arise from breakdown of albumin and preferential reuse of its amino acids in globulin-producing cells. Highly ^{35}S-labelled rat plasma was subjected to electrophoresis in a vertical column packed with cellulose. After 20 hr, about half the albumin had escaped into the

buffer above and was shown by paper-strip electrophoresis to be free of globulins. This was then injected into rats and, after 4 days, 10 per cent of the protein-bound radioactivity in the recipient's plasma was found to be distributed in α, β and γ globulins, the proportion increasing to half at 20 days. MAURER and MÜLLER's calculations showed that a much lower specific radioactivity of globulins would have resulted if all the label had come from free-plasma amino acids.

The writer and his colleagues in the course of studies with ^{14}C-labelled albumin and γ globulin separately in rabbits have always failed to observe any evidence of this kind of direct labelling of one protein by breakdown of the other. Several explanations of the discrepancy are possible. It may for example be emphasized that the detection of small amounts of globulins in the presence of albumin—amounts much less than are normally present in serum—is not easy by paper electrophoresis since up to 10 per cent of albumin is known to trail behind the main boundary. MAURER and MÜLLER's radioactivity record obtained from the electrophoresis paper strip of a test on the material they injected showed no sign of the albumin tail, and contrasts with a similar record from rat albumin obtained by CAMPBELL *et al.* (1956b). Also because of the short half-life of rat albumin and the much longer half-life of rat globulin a proportion of only 3 to 4 per cent total globulins in the recipient's plasma on the first day will become one of 10 per cent at 4 days for metabolic reasons alone. We may note also that no steps were taken by MAURER and MÜLLER to remove adsorbed labelled amino acids from the injected albumin—for example, by dialysis against unlabelled methionine and cystine.

VII. Is there a Mobile Intracellular Pool of Plasma Proteins?

Much evidence for the existence of such a pool has already been discussed. The case rests mainly on the following kind of experiment. As a result of fasting for 3 days a dog has its daily total urinary nitrogen output reduced from 7 g to 4 g (cf. DAFT *et al.*, 1938). It is then given 4 to 5 g nitrogen per day in the form of plasma protein administered intravenously along with a diet of adequate calorific value in the form of fat and carbohydrate (Cowgill diet). Urinary nitrogen excretion scarcely changes for the first 3 days, indicating complete retention of injected nitrogen, and subsequently it slowly increases. At the end of 10 days nitrogen excretion is still not quite equal to the sum of fasting plus daily injected nitrogen. Injections are discontinued at this point and fasting resumed when all the retained injected nitrogen is excreted over a period of days. The time lag associated with these changes is important.

When essentially the same experiments were carried out by MARTIN

and ROBISON (1922) on man but with oral instead of parenteral administration of protein, the same changes were apparent but with no preliminary period of total retention. Instead, abrupt changes of dietary nitrogen level were associated with immediate changes in the urinary level which settled down again to a constant value after 3 to 4 days. The obvious deduction from their experiments is that a protein store in the body has to adjust its reserves to changes in dietary nitrogen and requires 3 or 4 days to do so. The further deduction which has been widely made from experiments with transfused protein on the other hand is that during the period of sensibly complete nitrogen retention a similar reserve store is being increased by addition of whole plasma protein without preliminary breakdown to amino acids.

Some of the retained protein is obviously present in the circulating plasma. For example, in the typical dog experiment referred to, the plasma protein level slowly increased over the 10 days of injections from 7 g to 8·5 g /100 ml, which is however not sufficient to account for all the protein retained. The unwarranted implication is made in some earlier papers that all the remainder has passed into cells, its apparent disappearance being associated with parallel retention of phosphorus and potassium, these two elements with protein being regarded by ALBRIGHT *et al.* (1950) as essential constituents of protoplasm. By giving protein labelled with [^{14}C] lysine by vein, YUILE (1951) showed that nitrogen retention in this case was equivalent to protein retention, i.e. there was no deamination of the injected protein. The implication therefore is that the missing protein has in some way become associated with potassium and phosphorus to form cytoplasmic protein.

Clearly it should first be shown that the missing protein is not present in the extravascular space. This has seldom been done because adequate methods of measurement have not been available, but with the advent of satisfactory isotopic ones the situation must change. Since plasma and lymph volumes expand and contract with changes in plasma protein concentration (cf. ALLISON, 1950; ECKHARDT and DAVIDSON, 1950), and it is possible that lymph changes are disproportionately larger than plasma ones, such measurements will need to be made directly on animals or subjects under nutritional investigation and not inferred from similar measurements on animals in equilibrium.

Important observations were made on humans receiving daily transfusions of albumin by several clinical groups and especially by MEYER *et al.* (1947) and by ECKHARDT *et al.* (1948). The latter could account for up to 46 per cent of retained albumin in the increased plasma pool 16 days after starting daily injections of 40-g albumin, and clearly the remainder could have been accommodated in the extravascular space. When injections were stopped the excess protein in

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the plasma disappeared by an approximately exponential process with a half-life of 5 to 6 days, while FINK *et al.* (1944) observed a similar process in dogs also with a half-life of 5 days. ECKHARDT *et al.* (1948) draw attention to the fact that, if the elimination process continues to operate at the same exponential rate while lymph and plasma pools are expanding, then the mass of plasma protein broken down per day may be expected to increase until it is equal to the mass of protein injected per day. This would be in keeping with observed gradual increases in urinary nitrogen and plasma protein concentration and their asymptotic approach to constant values.

VIII. FATE OF INJECTED LABELLED PLASMA PROTEINS

If a reservoir of fully-formed plasma proteins exists inside certain cells it should be possible to demonstrate its existence by following protein radioactivities of organs and tissues for some days after injecting labelled plasma proteins into the blood stream. With this in mind YUILE *et al.* (1951) injected [^{14}C] lysine labelled total serum protein into three dogs with results which are shown in Table 1. One dog was

TABLE 1

(From YUILE, LAMSON, MILLER, and WHIPPLE, 1951)

Total C^{14} *Recovery after Intravenous Injection of Labelled Plasma Protein*

Source of C^{14}	*Experiment* 1	*Experiment* 2	*Experiment* 3
	Dog 48–106 49 *hr*	*Dog* 50–37 7 *days*	*Dog* 49–131 7 *days*
	Per cent administered C^{14}		
Tissues	38·77*	52·74	61·65
Plasma and perfusate	34·50	16·00	16·70
Plasma removed in sampling	5·80	5·60	7·90
Expired CO_2	5·20	6·73	4·92
Urine	0·50	2·50	1·31
Red blood cells	†	4·00	4·33
Total recovery	84·77	87·57	96·81

* Brain, bone marrow, skeleton, integument, colon, lymphoid, and aerolar tissues not sampled.

† C^{14} activity in 1 ml packed red blood cells insignificant.

viviperfused after two days and the other two after seven days with recovery of 80 to 90 per cent of estimated circulating plasma proteins. Assuming an L/P ratio of 1, and deducting total lymph activities from the total measured tissue activities shown in the table, the authors

estimate that 33 per cent and 41 per cent of injected ^{14}C-protein was present in intracellular form in the tissues in experiments two and three respectively after seven days. Similar approximate values were obtained from plasma specific-activity curves which showed that 46 per cent of proteins in lymph and plasma were eliminated over the period of 7 days. During this time 12 per cent could be accounted for in red cells, CO_2, and urine, and in blood samples leaving 34 per cent for "conversion into protoplasm." Because such a small proportion of the dose was excreted whereas the urine contained its normal complement of nitrogen, the authors concluded that tissue protein was being metabolized and injected labelled protein was being stored without breakdown.

Humphrey and McFarlane (1954b) tried to take this type of investigation a stage further by injecting ^{14}C-labelled normal and immune globulins into a rabbit 8 days before death, and Evans Blue 12 hr before death to act as an indicator of lymph. Their general aim was to estimate lymph protein in organs more directly than did Yuile (1951) and in addition by means of specific antigen-antibody precipitation to discriminate between ^{14}C-labelled tissue proteins and similarly labelled globulins which had penetrated into cells.

The broad distribution of total radioactivity found by them 8 days after injecting [^{14}C] phenylalanine-labelled globulins in the rabbit agreed remarkably closely with that obtained by Yuile *et al.* in dogs 7 days after injecting [^{14}C] lysine-labelled total serum proteins, suggesting that the fate of antibody globulin and total serum proteins is essentially the same. ^{14}C activity per gramme tissue carbon was highest in liver and adrenals, about two-thirds of this maximum value in the other viscera and lowest (one-third) in skin and muscle. Except in the case of skin which had a high lymph content, values measured with Evans Blue for proteins in lymph of organs proved to be not very different from the allowances used by Yuile *et al.* and later confirmed with several labels for muscle by Gitlin and Janeway (1954). By assuming that the ratio of Evans Blue concentration to total radioactivity or antibody concentration in lymph is the same as in the plasma at the same time it was possible to estimate amounts of radioactivity or precipitable antibody in tissue lymph and to subtract them from total values for the tissue. In general the amounts of radioactivity subtracted represented only a few per cent of total radioactivities except in the case of lymph glands (15 per cent) and of skin (nearly 30 per cent).

Without exception antibody present in tissue homogenates was fully accounted for as lymph protein, indicating that practically no antibody had penetrated into cells and remained intact. If this is true of other plasma proteins as well as immune globulins, it is probable that retained

injected proteins which have disappeared from lymph and plasma have been rapidly broken down, and their amino acids almost quantitatively reused to form tissue proteins. This breakdown could be imagined to take place to some extent in all cells and in such a way that the liberated amino acids were not allowed to mix with plasma amino acids, but there are singularly few arguments in favour of such a process and many can be cited against it. It seems altogether more probable that breakdown of plasma proteins, like their synthesis, is a function

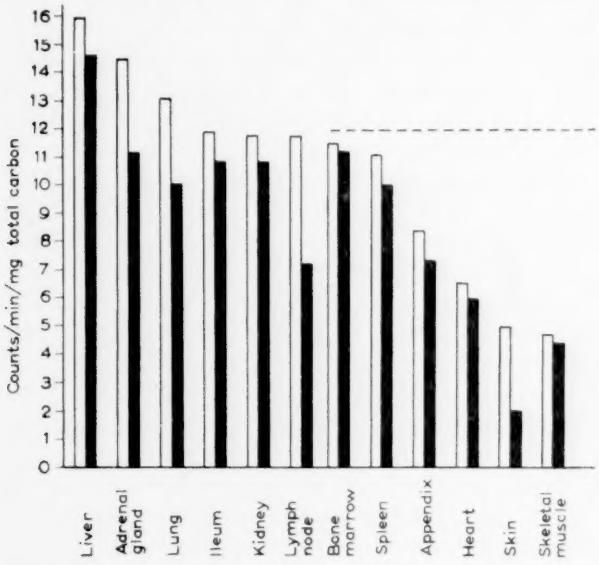

Fig. 13. Specific activities of nonantibody tissue protein carbon (open columns) in various perfused organs 8 days after transfusing 14-C antibody. Solid columns, specific activities after allowing for trapped lymph protein. The dotted line shows the specific activity of carbon in the plasma-free amino-acid fraction at death (from HUMPHREY and MCFARLANE, 1954b).

limited to certain cells, and that the amino acids liberated do in fact mix with those of the plasma pool. As previous considerations have shown, once they enter the plasma they may be expected virtually to disappear again in a short time because of the rapid turnover of amino acids there and the relatively enormous capacity of the tissue-protein pool.

An important consequence of this view is that tissue protein *specific* radioactivities which are a measure of the potential or intensity of the label cannot be higher than that of the plasma amino acids at the same time, whereas of course they could be much higher if they included even a small proportion of injected labelled protein. HUMPHREY and MCFARLANE therefore dialysed the rabbit plasma after death, adsorbed

the amino acids of the diffusate on Zeokarb, washed, eluted, and measured the specific activity of amino-acid carbon. The result (dotted line in Fig. 13) was equivocal in the case of intestine, kidney, bone-marrow and spleen, but showed clearly that the major proportion of body activity which is protein-bound in muscle and skin could all have come from secondary labelling by plasma amino acids. However, some part of the much smaller activities in liver, adrenals and lungs must have come directly from injected labelled protein, suggesting that these organs may have a special function in relation to plasma-protein breakdown. For example, ingested plasma protein awaiting digestion by phagocytic cells would explain the result.

The use of a single labelled amino acid such as [^{14}C] phenylalanine in this kind of work has certain disadvantages, especially as we have seen in relation to measurements of free amino acid specific activities. It may be mentioned here that HUMPHREY and MCFARLANE have repeated their investigation in all aspects using ^{14}C totally labelled plasma proteins and at the same time replacing Evans Blue as a lymph marker with ^{131}I-labelled total plasma proteins injected 20 hr before death. The distribution of specific radioactivity in the tissues at 6 days was substantially the same as in Fig. 13 except that the skin value was relatively twice as great. This is attributed to the fact that skin proteins contain very little phenylalanine. The plasma-free amino-acid specific activity had also practically the same relationship to specific activities of total carbon in the tissues. Lymph relative activities were unchanged, and once again precipitable antibody in tissue homogenates could all be accounted for as lymph protein. Tissue protein homogenates in this experiment were crudely separated with saline into soluble and insoluble fractions, the latter containing mainly structural protein. This always had a lower specific activity than the soluble protein. The largest factor of difference between the two (3 times) was found in liver in which the soluble protein carbon had a specific activity twice that of total carbon and more than twice that of plasma-free amino acids.

Therefore whereas the results exclude the presence of any large proportion of injected labelled plasma proteins inside cells, it is evident in the case of a few organs and especially of liver that some labelled protein is present intracellularly which could not have come from plasma amino acids. A liver cell which is producing albumin and also breaking down labelled plasma proteins clearly presents a favourable site for local transfer of the label to occur, but nevertheless this does not seem very probable. Alternatively, digestion may be a relatively slow stepwise process, and fragments of protein molecules may be present in the cell which have lost some of their distinctive characteristics including antigenicity but are still nondiffusible and

precipitable with trichloroacetic acid. These of course need only be present in a few phagocytic cells in order to raise the mean specific activity of tissue carbon above the level in plasma amino acids.

VIII. 1. *How is the plasma-protein level maintained?*

There is a widespread tendency to assume that withdrawal of plasma protein is made good by two processes separately or in combination. The rate of synthesis is increased and/or the rate of breakdown is diminished, and TARVER (1954) presents in tabular form a scheme for recognizing from measurements of plasma-protein specific activities which of the two processes is operative. Little attention has been given to a third and, in the writer's view, more likely possibility which is implied in observations referred to by ECKHARDT *et al.* (1948) and which may be formulated as follows.

HUMPHREY and MCFARLANE (1954a) showed that the exponential elimination rate of pneumococcus antibody globulin in the rabbit is independent of antibody concentration in the plasma over a wide range. Thus for an antibody level of 35 mg/ml, equivalent to 7·0-g antibody in lymph and plasma and an elimination rate of 12·5 per cent per day, 875 mg of antibody were broken down daily. In another animal with one-tenth this level and the same rate only 87·5 mg were broken down daily. The same considerations may well hold for other plasma proteins, but have never been shown to apply to synthetic processes. If we may assume on the contrary that rate of synthesis is independent of plasma-protein level, then the equilibrium level of a protein in the plasma could be determined by opposing factors of constant production on the one hand and variable breakdown determined by pool size on the other.

This self-stabilizing principle is in qualitative agreement with experimental results, but quantitative proof that it in fact determines plasma protein levels depends on the availability of information on extravascular pool sizes. Following addition or withdrawal of protein from the plasma pool, a protein-concentration difference exists between lymph and plasma. Subsequently this difference narrows and finally disappears by virtue of two simultaneous processes, viz. (*a*) transfer of protein between compartments, and (*b*) positive or negative balance of absolute production over breakdown occurring as already discussed mainly if not exclusively in proximity to the plasma. A comprehensive solution has not yet been described for the problems presented here, but one such should be possible. For a satisfactory explanation on this basis of the rapid rates at which normal plasma protein levels are known to be re-established, correspondingly rapid plasma protein breakdown rates are required. Rates which have been quoted, e.g. 12·5 per cent per day for rabbit antibody globulin or 20 per cent per day for rat

albumin, refer to protein in combined lymph and plasma pools. If breakdown is confined to the plasma compartment, corresponding rates become 31 per cent and 50 per cent respectively of protein in the plasma pool for L/P ratios of 1·5. The rates effective in re-establishing plasma protein levels must be somewhere between the lower and higher value in each case, and are consistent with observed periods of a few days which are required to make good the effects of even relatively large single transfusions or withdrawals.

IX. General Picture of Protein Metabolism

The classical physiology of Folin postulates a sharp distinction between a major part of urinary nitrogen, the excretion of which fluctuates with the dietary nitrogen level, and the remainder which like creatinine and uric acid is excreted independently of the diet. This constant fraction or so-called basal nitrogen excretion of an animal assumed to be in nutritional equilibrium and not using protein as a source of energy is visualized as arising from replacement of protein involved in "wear and tear" processes as distinct from free energy producing ones. It is extremely difficult to measure it accurately because the body has a disconcerting habit of "remembering" its dietary history for a surprisingly long time. Furthermore, the distinction between endogenous and exogenous nitrogen is not revealed as one might expect by any abrupt change in the course of urinary nitrogen excretion curves from animals on a rising level of dietary nitrogen when they pass from a state of net nitrogen loss to one of net gain (Allison, 1950).

These observations raise the question whether there is any essential chemical difference in the source of urea nitrogen in the fasting and in the replete animal. In other words are so-called wear-and-tear processes in the cell chemically divorced from other processes involving proteins and amino acids? On the contrary the consensus of tracer evidence favours the view that they are interdependent. Plasma proteins cannot be made, as we have seen, without tissue proteins also being turned over, and neither can they be broken down to amino acids without changes taking place in expired CO_2. The most satisfactory arrangement which appears also to be the simplest, is one (cf. Fig. 12) consisting of an intracellular amino-acid pool in close association with plasma-free amino acids and in similar dynamic equilibrium with the amino acids of all protein systems in the cell. This implies that a change in the mass of protein or amino acids in any one pool must inevitably affect in some degree the masses in all the others.

On this basis a cell which is replete in nitrogen must be visualized as leading a fuller life in all aspects of its cellular biochemistry than a fasting one, with few if any qualitative differences between the two. Much evidence of a nonisotopic character favours this picture. Histo-

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logically the replete cell is enriched over the fasting one in all its structures—nuclear, cytoplasmic and granular (ELMAN and HEIFETZ, 1941), while MILLER (1948) has shown that its enzymic activities also increase in parallel with nitrogen acquisition. This interdependence of systems within cells means also by virtue of the common plasma amino-acid pool an interdependence of cell systems throughout the body. Thus ablation of two-thirds of the rat liver is followed by rapid regeneration using nitrogen which may come in part from other tissues (ROBERTS, 1953). In the fasting animal liver protein is always most heavily sacrificed (40 per cent in 7 days according to ADDIS *et al.*, 1936) and plasma protein is conserved so that only 3 per cent of the total loss is borne by it (WEECH, 1938). On this view neither plasma protein nor any single kind of cytoplasmic protein can be taken to constitute the mobile protein store of the body, but all of them participate in some degree according to the severity of the emergency.

If these views are correct it follows that normal metabolic behaviour cannot reasonably be deduced from studies on nutritionally disturbed animals, and indeed that the results of such studies may well be misleading. For example, it is possible from transfusion studies on depleted animals as we have seen to conclude that globin of haemoglobin is a specific precursor of plasma proteins (WHIPPLE *et al.*, 1945), or that plasma proteins are precursors of the matrix of bone in cases of osteoporosis (ALBRIGHT *et al.*, 1950) both of which are highly unlikely on grounds of amino-acid composition. Isotopic methods in spite of the difficulties of interpretation which have been discussed here and to which FISHER (1954) also draws attention seem to offer the only possibility of studying the organism as a whole under practically equilibrium conditions, and nothing short of this would appear to be really satisfactory.

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THE USE OF ISOTOPES IN THE STUDY OF INTERMEDIARY METABOLISM

H. R. V. Arnstein and P. T. Grant

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THE USE OF ISOTOPES IN THE STUDY OF INTERMEDIARY METABOLISM

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I. Introduction

Intermediary metabolism may be defined as the chemical reactions by which living organisms utilize food for energy and for the synthesis of compounds essential for their continued life. The classical methods used to study intermediary metabolism (see Fruton and Simmonds, 1954) have all suffered from the disadvantage that the experimental conditions differed in one way or another from the normal physiological state of the organism. More recently, the use of "biochemical" mutants (Spiegelman and Landman, 1954) and the application of isotopes have made possible studies under essentially normal conditions. The former method is, however, restricted to micro-organisms so that in most cases the isotopic tracer technique is the only satisfactory method for studying metabolic pathways *in vivo*. Moreover, this technique is often more convenient than the classical methods, and sometimes essential for the study of metabolic reactions in isolated tissue preparations or enzyme systems.

In the present review, an attempt has been made to discuss mainly the more recent work which illustrates the scope of the isotopic-tracer technique. The design of biological experiments and the interpretation of isotopic data have been emphasized rather than the importance of the results themselves.

II. The Assay and Detection of Isotopes used as Biological Tracers

The food of a living organism is a complex mixture of compounds containing mainly the elements of carbon, hydrogen, oxygen, nitrogen, sulphur and phosphorus, and the isotopes of these elements have been extensively used as tracers to follow the intermediary metabolism of such compounds. The present review, therefore, deals only with the assay of the naturally occurring stable isotopes ^{13}C, ^{15}N, ^{18}O, and deuterium (^{2}H), and the unnatural radioactive isotopes ^{14}C, ^{32}P, ^{35}S, ^{131}I and tritium (^{3}H).

II. 1. *Stable isotopes*

Stable isotopes of an element occur in almost constant proportions (Table 1). For example, the ratio of ^{15}N to ^{14}N in normal nitrogen is

38:9962, and the normal abundance of ^{15}N is, therefore, 0·38 atom per cent. Artificially enriched samples may contain up to 60 atom per cent ^{15}N or (60 — 0·38) atom per cent ^{15}N excess over the normal abundance, atom per cent rare-isotope excess being a useful expression for calculations involving stable isotopes (SCHOENHEIMER and RITTENBERG, 1939).

The ratio of isotopes present in a sample is usually determined in a mass spectrometer. Thus, the ratio $^{15}N:^{14}N$ is determined from the intensities of the ion current at the peaks of mass 29(^{15}N, ^{14}N) and 28(^{14}N, ^{14}N), peak 30(^{15}N, ^{15}N) being usually too small for accurate measurement. Similarly, ^{13}C is estimated from the ratio of peaks of mass 44($^{12}CO_2$) and 45($^{13}CO_2$). The theory and practice of mass spectrometer analyses has been discussed recently by BARNARD (1953).

Several methods utilizing differences in the physical properties of D_2O and H_2O are available for the analysis of deuterium. For routine analysis, density is the only useful property, and the "falling drop" method is described in detail by GLASCOCK (1954). The cheapness and simplicity of this procedure, which has also been used to estimate ^{18}O (DOLE, 1952) may, however, be largely outweighed by the fact that relatively large water samples (about 100 mg) are required, and the sensitivity is low, $\pm$ 0·02 atom per cent D (cf. Table 1). The difficulties involved in the determination of deuterium by mass spectrometry have been discussed by WASHBURN, BERRY and HALL (1953).

An inherent limitation to the use of stable isotopes as biological tracers is the relatively narrow range between the highest concentration which can be obtained (Table 1) or safely administered to a living organism (see p. 184) and the lowest concentration which can be estimated with some accuracy. For example, according to the sensitivity limits of the mass spectrometer given in Table 1, the maximum detectable dilution of a sample containing 70 atom per cent ^{13}C excess would be 5000 fold, i.e. 0·014 $\pm$ 0·01 atom per cent ^{13}C excess. The significance of such a low excess of ^{13}C over normal may, however, be doubtful, because the natural abundance ratio may vary slightly according to the source (see THODE, 1949). It is thus advisable to design biological experiments so that the measurement of almost normal isotope ratios is avoided. A 350-fold dilution, for example, would give a sample containing 0·2 atom per cent ^{13}C excess, which can be estimated with an accuracy of $\pm$ 5 per cent, whilst the possible error due to variations in the natural-abundance ratio would be negligible.

II. 2. *Radioactive isotopes*

II. 2. 1. *Quantitative assay of radioactivity*

Natural organic compounds do not contain radioactive isotopes except for minute traces of ^{14}C and tritium (^3H) which are produced by cosmic-ray bombardment of the atmosphere (LIBBY, 1955).

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TABLE 1

Stable Isotopes used as Tracers in Metabolic Experiments

Isotope and mass number	*Natural abundance ratios**	*Abundance commercially obtainable*	*Sensitivity of mass spectrometric analyses*
$^{1}H:^{2}H$ (deuterium)	99·985:0·015	About 100 atom % D excess	± 0·002 atom % D†
$^{12}C:^{13}C$	98·90:1·10	,, 70 atom % ^{13}C excess	± 0·010 atom % ^{13}C†
$^{14}N:^{15}N$	99·62:0·38	,, 60 atom % ^{15}N excess	± 0·003 atom % ^{15}N†
$^{16}O:^{17}O:^{18}O$	99·76:0·04:0·20	,, 6 atom % ^{18}O excess	—‡

* Compiled from data given by BARNARD (1953).
† Quoted from ARNSTEIN and BENTLEY (1950).
‡ See COHN and DRYSDALE (1955).

TABLE 2

Radioactive Isotopes used as Tracers in Metabolic Experiments

Isotope	*Type of radiation*	*Half-life*	*Maximum energy of radiation* (MeV)		*Specific activity commercially obtainable*
			Particles	*γ rays*	
^{3}H (tritium)	β^-	12·46 years	0·018	—	220 mc/ml water
^{14}C	β^-	5568 years*	0·15	—	1–9 mc/mmole
^{32}P	β^-	14·30 days	1·70	—	1–10 mc/mmole
^{35}S	β^-	87·10 days	0·17	—	1–10 mc/mmole
^{131}I	β^-,γ	8·0 days	mainly 0·6, 0·25	mainly 0·64, 0·36 0·28, 0·08	5–200 mc/mmole

The data in this table are compiled from Catalogue No. 3, Radioactive materials and stable isotopes (1954), published by the Atomic Energy Research Establishment, Isotope Division, Berks., England.

* LIBBY (1955), has reviewed the somewhat controversial evidence on the half-life of ^{14}C and has concluded that the most accurate value is 5580 ± 45 years.

During the decay process of biologically important radioactive isotopes charged particles are emitted which may be accompanied by electromagnetic radiation (Table 2). It is important to realize that, for various reasons, which are discussed later, only a fraction of the absolute number of disintegrations occurring in the sample per unit of time can be recorded. This fraction is termed the overall efficiency of a counter for a particular isotope. It is, therefore, usual to compare the specific activity of all samples under identical conditions with a suitable reference standard of known absolute specific activity, such as may be obtained from the Radiochemical Centre, Amersham, and similar establishments.

The unit of radioactivity, the curie (c), is defined as the quantity of any radioactive nuclide in which the number of disintegrations per second is $3{\cdot}7 \times 10^{10}$ (PANETH, 1950). For biological tracer studies, this unit is too large, the millicurie (mc) and the microcurie (μc) being convenient subdivisions. The relative radioisotope concentration in a compound is termed the specific radioactivity, i.e. μc/mg, μc/mmole, etc.

All samples can be assayed only in the presence of a background of counts, which is characteristic of the counting device and its environment. The true counting rate of the sample is obtained by subtracting the background count, measured when the counter is operating with no radioactive source present, from the total observed counting rate of the radioactive sample. Radioactive decay is a random process so that statistical errors due to background and sample fluctuations are inherent in radioactive assay. The statistical treatment of counting data is discussed by CALVIN *et al.* (1949) and TAYLOR (1951).

Assay of ^{14}C. In the simplest and most convenient method, a solid sample may be counted in a thin end-window Geiger counter, where the sample is mounted outside the gas-filled chamber (GEIGER-MÜLLER tube), and immediately below the window through which radiations may pass into the chamber. With the thinnest available window (about 1 mg/cm^2 of mica), the overall counting efficiency is about 1–2 per cent of the disintegration rate of ^{14}C.

The insertion of the solid sample inside the chamber, through which a slow flow of a suitable gas mixture is maintained, eliminates window absorption, so that the overall efficiency is increased. Moreover, the "dead time" during which a counter is not capable of recording an ionizing event is greatly reduced (Table 3), and much higher specific activities can be accurately assayed. The more versatile nature of this instrument, working either in the Geiger or proportional region, has made it increasingly popular for the radioassay of solid samples (see GARROW and PIPER, 1955).

The main advantage of the direct radioassay of solid organic compounds is that, in addition to the usual criteria, radiochemical purity

TABLE 3

Relative Counting Rates of Various Radioactive Isotopes

	TYPE OF COUNTER		
	End-window Geiger-Müller	*Windowless Geiger-Müller gas-flow* (99% He + 1% isobutane)	*Windowless proportional gas-flow* (90% A + 10% CH_4)
^{14}C	0·031	1·0	1·1
^{35}S	0·030	1·0	1·2
^{32}P	0·240	1·0	1·7
^{131}I	0·130	1·0	1·2
Average background (counts/minute)	17·0	31·0	72·0
Dead time (μ seconds)	236·0	150·0	1·28

Data in this table were obtained from the paper by GRAF, COMAR, and WHITNEY (1951). The relative counting rates for samples of each isotope have been extrapolated to "zero mass" and are not corrected for background. Due to the window thickness (3·5 mg/cm²) and the distance of the sample from the window (2 cm), the relative counting rates of the low-energy radiation of ^{14}C and ^{35}S in this end-window Geiger-Müller counter are lower than would be obtainable with more recent instruments. It is now usual to mount the sample closer to an end window of only 1–2 mg/cm² thickness.

can be established by repeated crystallization and radioassay until the radioactivity of the sample is constant. Subsequently, the pure compound can be used for the location of isotope within the molecule by degradation. Some organic compounds are, however, extremely difficult to prepare in uniform layers in a reproducible manner due to their granular or hygroscopic nature. In these circumstances it is usual to convert the compound into a suitable derivative or to combust the sample and radioassay the CO_2 produced as $BaCO_3$ (ABRAMS and CLARK, 1951). $Ba^{14}CO_3$ samples must not be exposed to CO_2 in a moist atmosphere because of interchange between carbonate and CO_2 (ARMSTRONG and SCHUBERT, 1947).

For the preparation of solid samples, the compound is compressed into an even layer of known surface area (usually 1 cm^2 or larger) in a rimmed disk or shallow cup (POPJÁK, 1950; BURR and MARCIA, 1955). Alternatively, a suspension may be evenly deposited by filtration (SCHWEITZER and STEIN, 1950) or centrifugation (HUTCHENS, CLAYCOMB, CATHEY and VAN BRUGGEN, 1950) on to a filter-paper circle supported on a perforated disk.

Because of the low energy of the β particles emitted by ^{14}C, an appreciable proportion of the radiation from samples exceeding approximately 5 mg/cm^2 is absorbed in the upper layers of the sample itself (self-absorption), and the observed radioactivity is no longer proportional to the mass of the sample. At about 20 mg/cm^2, the radioactivity from the bottom layer is completely absorbed by the upper layers, and further increase of the sample mass above this amount does not change the observed radioactivity. Such samples are termed as being of "infinite thickness." If less than this amount of a compound is available, the observed radioactivity may be corrected to an "infinite thickness" value by means of an experimentally determined relationship between radioactivity and sample thickness (ARMSTRONG and SCHUBERT, 1948). The counting rates of infinitely thick samples of the same compound are proportional to their specific activities. In addition to the radiation emitted by the upper layers of the sample within the solid angle contained by the source and the end window, the observed radioactivity includes β particles reflected into the solid angle mainly by collision with the sample material. This effect is termed "backscattering," and its magnitude increases with increasing average atomic number of the radioactive compound (CALVIN *et al.*, 1949). For samples of compounds whose average atomic numbers do not differ markedly, the backscattering component is constant and the observed counting rates are directly comparable. Compounds with an exceptionally high average atomic number (e.g. $BaCO_3$, iodoform) exhibit appreciable backscattering, the extent of which depends on the precise conditions of radioassay, such as counter-geometry, the nature

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of the disk or cup containing the sample and the thickness of the end window of the counter. These considerations probably account for the fact that the observed radioactivities of $BaCO_3$ samples have been reported to exceed those predicted by 34 per cent (TOPPER and HASTINGS, 1949), 26 per cent (CRANE and BALL, 1951), 5 per cent (ARNSTEIN and BENTLEY, 1953), and 5 per cent for 5 mg/cm^2 samples counted in a gas-flow windowless proportional counter (KARNOVSKY *et al.*, 1955; see also NELSON and KROTKOV, 1955). Consideration of errors due to backscattering may be avoided by counting all samples in the same chemical form (i.e. $BaCO_3$), but many workers perfer to count organic compounds directly and apply such corrections as may have been found necessary in their own particular procedure of radioassay.

In biological experiments it is not uncommon to isolate only a few milligrammes of a pure intermediate. In this case the compound may be deposited from solution by evaporation on circles of microscope lens paper mounted on disks (CALVIN *et al.*, 1949). Such samples are termed "infinitely thin" or of "zero mass," and this technique has been used to count fatty acids (ENTENMAN, LERNER, CHAIKOFF and DAUBEN, 1949) and *N*-dinitrophenylamino acids (ASKONAS, CAMPBELL, GODIN and WORK, 1955; FLAVIN and ANFINSEN, 1955). Infinitely thin samples do not require self-absorption corrections. For example, the observed radioactivities of a standard sample of dinitrophenyl-[α-^{14}C] glycine were proportional to the sample weight in the range 20–200 μg/cm^2, the counting rate of the 200 μg sample being about 1/50 of that obtained with an "infinitely thick" sample of the same material (HENRIQUES, HENRIQUES and NEUBERGER, 1955). The backscattering component in the observed radioactivities of infinitely thin samples is almost entirely due to reflection of β particles by the sample mount (GLENDENIN and SOLOMON, 1950). For this reason, the observed radioactivities of different compounds should be directly comparable, provided the samples are mounted in an identical manner.

Small biological samples (2–15 mg) may be usually counted with higher efficiency as $^{14}CO_2$ in a gas-phase counter (BRADLEY, HOLLOWAY and MCFARLANE, 1954; GLASCOCK, 1954; SINEX *et al.*, 1955). $^{14}CO_2$ from the combusted sample is mixed with another suitable gas and introduced directly into the counting chamber. In this way almost all the radioactive molecules are within the sensitive counting volume, and some 98 per cent of the disintegrations may be detected (SINEX *et al.*, 1955). Moreover, self-absorption and backscattering are negligible. This technique is advantageous because of its overall efficiency and the small amount of sample required, but gas-phase counting is not always superior to solid counting. For example, an "infinitely thin" sample (1 mg) of ^{14}C-labelled liver protein gave 16 counts/minute, of which 7

were background, in a windowless gas-flow proportional counter, while a similar sample counted as $^{14}CO_2$ in a gas-phase instrument gave 36 counts/minute, of which 18 were background (GARROW and PIPER, 1955). Since the accuracy of counting increases with the ratio of total counting rate to background, there is little difference between the two types of counter under these conditions. If the sample sizes were larger, however, the gas-phase counter would be preferable since the observed radioactivity would increase linearly with increasing sample weight, whereas with the solid counter the increase in radioactivity would be less, due to self-absorption.

Other instruments which are of more limited application for the routine radioassay of ^{14}C in biological samples are the ionization chamber (BAKER, TOLBERT and MARCUS, 1955; COLLINS and ROPP, 1955) and the scintillation counter (STRANKS, 1956; WAGNER and GUINN, 1955).

Assay of ^{35}S. The maximum energy of the β particles emitted by ^{35}S is similar to that of ^{14}C, so that the same technique and methods may be used. The solid organic compound may be counted directly, or it may be oxidized to inorganic sulphate and precipitated as benzidine sulphate (TARVER, 1951; GAITONDE and RICHTER, 1955).

The self-absorption and backscattering of ^{35}S are similar to those of ^{14}C (GLENDENIN and SOLOMON, 1950). Due to the relatively short half-life of the isotope (Table 2), radioactivities observed over a period of days or weeks must be corrected for isotopic decay. This is most conveniently done by using a portion of the original ^{35}S-labelled material as a reference standard.

The radioassay of ^{35}S in the gas phase as $H_2{}^{35}S$ (BERNSTEIN and BALLENTINE, 1950) or $^{35}SO_2$ (NORRIS, 1952) and in the liquid phase (WALSER, REID and SELDIN, 1953) have also been reported.

Assay of tritium. Until a few years ago, tritium was not extensively used as a tracer in biological experiments, chiefly because of the difficulties involved in its radioassay. It is the weakest radioactive source known, having approximately 1/10 of the maximum energy of the β particles of ^{14}C. Owing to this fact, the counting of solid samples by a windowless gas-flow proportional counter (EIDINOFF and KNOLL, 1950) or scintillation counter (HAYES and GOULD, 1953) is less sensitive than the radioassay of ^{14}C in an endwindow Geiger-Müller counter. The only efficient method is the direct gas-phase assay of tritium. The gases containing tritium which are preferred for counting are hydrocarbons such as methane (ROBINSON, 1951), butane (GLASCOCK, 1954), or acetylene (WING and JOHNSON, 1955), although water vapour (PACE, KLINE, SCHACHMAN and HARFENIST, 1947) and hydrogen gas (BERNSTEIN and BALLENTINE, 1950) have also been used. The disadvantage of hydrogen gas or water vapour is their absorption on the

interior surfaces of the counting chamber and the difficulty of removing such contamination.

Methods for the radioassay of tritium are listed by THOMPSON (1954), and a description and discussion of most of them is given by GLASCOCK (1954).

Assay of ^{32}P. The maximum energy of the radiation of ^{32}P is about 10 times that of ^{14}C (Table 2), so that the radioassay of ^{32}P as a solid sample in an end-window Geiger counter is more efficient because there is little absorption of the high-energy β particles by the window material (see Table 3). It has been estimated that the overall efficiency of the thin-end-window Geiger counter for solid samples (strychnine phosphomolybdate or magnesium ammonium phosphate) is about 38 per cent (CLARKE, 1952). Self-absorption is not appreciable for samples less than about 50 mg/cm^2 (KAMEN, 1947), and backscattering corrections are usually avoided by comparing the radioactivities of all samples in the same chemical form under identical conditions of radioassay.

The radiation of ^{32}P readily penetrates thin glass, and for this reason the isotope is most often radioassayed by dipping an all-glass counting chamber into the radioactive solution. Liquid counters (HEVESY, 1948; VEAL, 1948; ROSE and EMERY, 1951) are obtainable commercially. The overall efficiency of such a liquid counter for ^{32}P (10 ml sample) has been estimated as 5 per cent (VEAL, 1948).

The short half-life of the isotope (Table 2) makes decay corrections necessary. This is most conveniently done by making a reference standard of some of the original ^{32}P-labelled material used in the experiment.

Assay of ^{131}I. Compared with ^{14}C, ^{35}S and ^{32}P, ^{131}I has not been used extensively as a tracer in studies of intermediary metabolism and radioassay procedures for this isotope will not be considered in detail.

BRUNER and PERKINSON (1952) have compared the relative efficiency and sensitivity of nine different types of instrument for the assay of ^{131}I. They concluded that windowless gas-flow proportional counting of a solid sample is preferable to scintillation counting. More recently, however, improved scintillation techniques (BELCHER, 1953; HAIGH, 1954) permit solid and liquid counting with improved efficiency.

II. 2. 2. *Qualitative radioactive assay*

In many instances metabolic intermediates are unknown or occur only in trace amounts. A simple method of detecting and estimating radioactive intermediates is to separate crude-tissues extracts by two-dimensional chromatography, and locate the radioactive areas by autoradiography. The intermediates can then be identified by comparison with the chromatographic behaviour of known compounds. The amount and radioactivity may be estimated from the size and

density of the spot on the x-ray film, or alternatively as infinitely thin samples after elution from the paper. In this manner some of the photosynthetic products of $^{14}CO_2$ fixation by algae (Benson *et al.*, 1950) and the metabolic products of ^{14}C-acetate incubated with liver slices (Katz and Chaikoff, 1954) have been identified. Indeed, the presence of an unknown ^{131}I-containing spot in an extract of thyroid tissue led to the discovery of 3:5:3′-triiodothyronine as a normal constituent of thyroid tissue (Gross and Pitt-Rivers, 1953).

The radioactivity on paper chromatograms (usually one-dimensional) may also be automatically detected by moving the strip slowly by mechanical means under the end-window of a Geiger-Müller counter (Winteringham, Harrison and Bridges, 1953; Carleson, 1954) or a windowless gas-flow proportional counter (Bangham, 1956). Such automatic scanning devices are also commercially available.

III. The Preparation of Isotopically Labelled Compounds

Direct neutron irradiation of suitable compounds has been used to prepare [^{35}S] cystine (Ball, Solomon and Cooper, 1949), [^{14}C] glucose or benzoic acid (Wolfgang, Rowland and Turton, 1955) and [^{60}Co]-vitamin B_{12} (Anderson and Delabarre, 1951), but up to the present the induced radioactivity has been too low to be of practical value. Accordingly, labelled compounds of high specific activity are normally chemically synthesized or biosynthesized.

III. 1. *Chemical synthesis*

Several recent reviews deal exhaustively with the preparation of labelled compounds (Weygand and Simon, 1954; Thomas and Turner, 1953; Arnstein and Bentley, 1950). In addition many of the experimental procedures are described in some detail in an American Atomic Energy Commission Supplement (1952), by Calvin *et al.* (1949) and Catch (1953).

III. 2. *Biosynthesis*

It is not always practicable to prepare isotopic compounds by chemical means, and biosynthetic reactions have been frequently employed. The method is of general application for the uniform labelling of compounds such as [^{14}C] sugars and amino acids, or for the labelling of molecules containing only one atom of an element to be traced (Table 4). The usual design of a biosynthetic preparation is to obtain as high a yield of product as possible consistent with a minimum dilution of the added isotope. For example, green leaves were first depleted of starch before being allowed to photosynthesize glucose from $^{13}CO_2$ under previously determined optimum conditions (Livingston and Medes, 1948).

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TABLE 4

Some Isotopic Compounds Prepared by Biosynthesis

Isotope administered	*Biosynthetic system*	*Isotopic product and yield based on total isotope administered*	*Type of labelling*	*References*
^{14}C-formate, glycine or CO_2	Whole animal (pigeon)	Uric acid (from 3–60%) in urine	Preferential incorporation into some carbon atoms	Karlsson and Barker (1949)
^{14}C-acetate	Animal tissue (rat liver slices)	Cholesterol (10%) and fatty acids (21%)	Not determined	Brady and Gurin (1951)
$^{32}PO_4^{---}$	Animal tissue (sheep heart)	Adenosine triphosphate (80%)	—	Hems and Bartley (1953)
^{14}C-glucose	Enzyme system (maltose, phosphorylase)	Maltose (57%)	—	Fitting and Putman (1952)
$^{14}CO_2$	Plants (green leaves)	^{14}C-glucose, fructose and sucrose (70%)	Uniformly labelled	Putman and Hassid (1952)
$^{14}CO_2$	Micro-organisms (algae)	L-amino acids (20% in protein)	Uniformly labelled	Catch (1954)
$^{32}PO_4^{---}$ and $^{35}SO_4^{---}$	(yeast)	Coenzyme A	—	Sen and Leopold (1955)
$^{35}SO_4^{--}$	(yeast)	L-cystine (20%) and L-methionine (40%)	—	Niklas (1955)
$^{35}SO_4$	(fungi)	Penicillin (20%)	—	Pollock and Perret (1953)

Procedures for specific labelling, particularly the carbon atoms of a molecule, are, however, dependent on exceptionally favourable circumstances (Table 4). For example, the liver glycogen of the rat may be depleted by fasting and glycogenesis then stimulated by feeding glucose or lactate and at the same time injecting $NaH^{14}CO_3$. In this way about 2·5 per cent of the added isotope can be recovered as liver glycogen, in which the glucose units contain most of the radioactivity (about 98 per cent) at $C_3 + C_4$ (SHREEVE, FEIL, LORBER and WOOD, 1949). TOPPER and HASTINGS (1949) incubated rabbit liver slices with $NaH^{14}CO_3$ and found no activity in positions other than $C_3 + C_4$ of glucose obtained from glycogen.

III. 3. *Criteria of purity of isotopically labelled compounds*

III. 3. 1. *General considerations*

While ordinary chemical tests such as melting point, etc. are indicative, the only satisfactory criterion of purity for labelled compounds is the complete association of the isotope with the compound in question. Substances containing stable isotopes may be recrystallized from different solvents until the atom per cent rare isotope excess remains constant with repeated purification. Radiochemical purity can be established in a similar manner. There are cases, however, when even this criterion is inadequate. The pipsyl derivatives of some amino acids, for example, tend to coprecipitate from solution (KESTON, UDENFRIEND and CANNON, 1949). Radioautography or scanning of paper chromatograms can be used to determine whether all the radioactivity is associated with the labelled compound (PUTMAN and HASSID, 1952; OSTWALD, ADAMS and TOLBERT, 1952).

Quantitative estimates of purity can also be made by degradation, when the sum of the isotope content of the fragments should agree with that of the whole compound (BOOTHROYD *et al.*, 1955), or by isotope dilution. In the latter case, a known amount of the isotopic compound is thoroughly mixed in solution with a known amount of its nonisotopic counterpart. The compound is then purified, usually by crystallization or chromatography, and its isotope content determined. If the original isotopic sample was pure, the dilution observed in the isotope content of the reisolated sample should be the same as the mass-dilution factor. An important advantage of this method is that the sample may be rigorously purified until its isotope content remains constant. For a fuller discussion of the principles of isotopic dilution analysis see p. 204.

It may be necessary to establish the radiochemical purity of radioactive compounds stored for prolonged periods prior to their use as tracers in biological experiment, since some compounds decompose

under the influence of their own radiation (LEMMON, 1953). The most striking example is [methyl-^{14}C]choline chloride, 63 per cent of which was destroyed in about one year, even though the anhydrous sample was stored in the dark in sealed evacuated ampoules. The main products of the decomposition were [^{14}C]trimethylamine and unlabelled acetaldehyde (TOLBERT *et al.*, 1953; see also LEMMON, PARSONS and CHIN, 1955). [^{14}C]Methanol (8–10 mc/mmole) has also been found to decompose (SKRABA, BURR and HESS, 1953) and in this case hydrogen, methane, ethylene glycol, glycerol and mannitol, were present after storage. More recently, thioctic acid (6:8-dithiooctanoic acid), 200 μc^{35}S/mg, has been claimed to undergo self-decomposition at a rate of about 0·3 per cent per day (ADAMS, 1955). Other highly radioactive compounds, such as some amino acids, apparently decompose only to a negligible extent (LEMMON, 1953).

III. 3. 2. *The optical purity of stereoisomers*

The stereochemical specificity of many biological reactions makes it desirable that a labelled stereoisomer be stereochemically as well as analytically pure with respect to the isotope employed. The criteria of isotopic purity of such asymmetric compounds may be illustrated by reference to the optical enantiomorphs of the α amino acids.

Optical purity is most often determined by the optical rotation of amino-acid solutions in a polarimeter, but this method does not detect small amounts of a contaminating enantiomorph (GREENSTEIN, 1954). Optically specific enzymes which oxidize or decarboxylate the contaminant isomer are extremely sensitive, 0·1 per cent being detectable, but are not applicable to all amino acids (GREENSTEIN, 1954).

Methods are available which are based directly on isotopic rather than on optical purity. Thus, highly radioactive benzyl-DL-[^{35}S]cysteine was diluted 66 fold with pure unlabelled benzyl-L-cysteine, and the mixture recrystallized from enough solvent to retain all benzyl-DL-cysteine in the mother liquor. The resulting crystals, mainly diluted benzyl-L-[^{35}S] cysteine, were repeatedly recrystallized to a constant radioactivity which was the same as that calculated from the degree of dilution of the L-enantiomorph. Since the radioactivity of the D-enantiomorph was 66 times that of its L-counterpart, traces of the D-contaminant would have been readily detectable (WOOD and GUTMAN, 1949). This procedure may be generally applicable to the isolation of radioactive stereoisomers provided that the solubility of the racemic derivative is not markedly affected by the presence of a large excess of one of the optically active forms. In the particular case of lysine picrate, coprecipitation may account for the failure to obtain L-[^{14}C]lysine in a reasonable state of purity (ARNSTEIN, HUNTER, MUIR and NEUBERGER, 1949).

The criterion of isotopic purity of a labelled enantiomorph would obviously be satisfied if the contaminating isomer contained only an insignificant amount of the isotope. The isotopic dilution procedure was first used for this purpose by SCHOENHEIMER, RATNER and RITTENBERG (1939). The separation of the salts of a racemic acid (an *N*-substituted α amino acid, in this case) with an optically active base by one recrystallization is usually incomplete. The mother liquor in addition to containing almost all of the soluble diastereoisomeric salt is saturated with respect to the insoluble "contaminating" salt. When the original racemic amino acid is isotopically labelled, the insoluble salt present in the mother liquor can be replaced by crystallization after addition of a large excess of its unlabelled counterpart. By this procedure, the total amount of contaminating diastereoisomer in solution remains unchanged (i.e. the mother liquor is still saturated with respect to the insoluble salt), but the amount of isotope contained by the contaminant can be reduced to any desired extent by repeating the procedure. Thus, if M is the mass of labelled contaminating diastereoisomer of isotope content x originally present, and M' is the mass of unlabelled contaminating diastereoisomer added each time, then the isotope content y of the contaminant remaining in solution after n recrystallizations from a constant volume is given by the formula $y = x\left(\frac{M}{M+M'}\right)^n$. Since x and y can be measured and M' is known, it is possible to use this equation, where $n = 1$, to calculate the mass of contaminant diastereoisomer originally present, i.e. $M = \frac{M'y}{x-y}$. By this procedure a sample of the brucine salt of *S*-benzyl-*N*-formyl-L[β-^{14}C]cysteine was found to contain 7 per cent of the D-diastereoisomer, although only 0·1 per cent of the isotope was associated with the contaminant (ARNSTEIN and GRANT, 1954b).

IV. THE DEGRADATION OF ISOTOPICALLY LABELLED COMPOUNDS

While the incorporation of any isotope into the product of a biological system shows that it is metabolically related to the added labelled compound, the distribution of ^{14}C in the product is frequently indicative of the reactions which may have been involved. This aspect of the tracer technique requires procedures whereby specific portions of the molecule, preferably one-carbon fragments, may be isolated in a form suitable for isotope analysis.

Degradative methods must be proved to yield unequivocal results, and this is usually accomplished by checking the procedure with the compound specifically labelled by chemical synthesis. For example,

in order to prove that iodoform is only derived from the methyl group of acetaldehyde, $CH_3{}^{14}CHO$ was synthesized and treated with hypoiodite. Of the 1210 counts/min of ^{14}C in the acetaldehyde, none appeared in the iodoform (EHRENSVÄRD, REIO, SALUSTE and STJERNHOLM, 1951). Alternatively, the results of several presumably specific degradation procedures may be compared. In this way, the validity of a method whereby all six carbon atoms of glucose are obtained individually as $BaCO_3$ was confirmed by comparison with the results from partial degradation procedures (BERNSTEIN, LENTZ, MALM, SCHAMBYE and WOOD, 1955). The essential reactions of this particular procedure are given in Fig. 1. Apart from the initial bacterial fermenta-

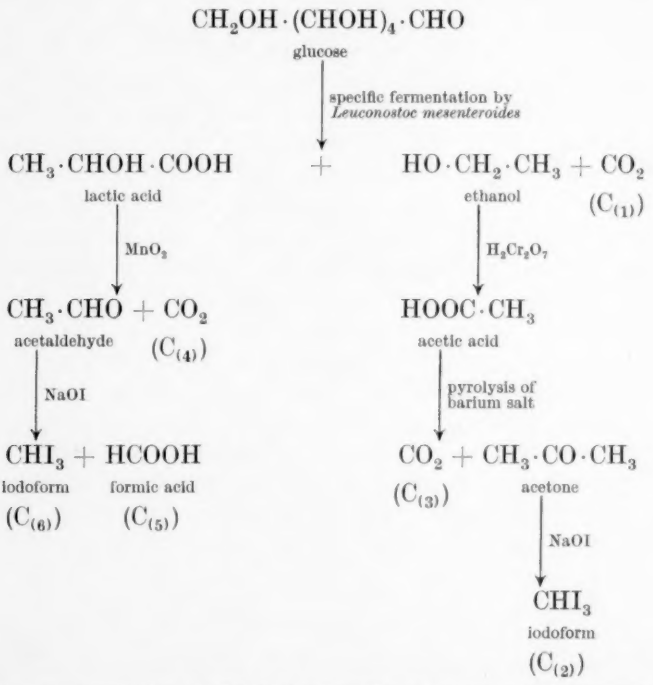


Fig. 1. Degradation of labelled glucose. From the paper by BERNSTEIN *et al.* (1955).

tion of glucose, the reactions are typical of those usually employed for the specific carbon by carbon degradation of small isotopically labelled molecules.

For direct gas counting of ^{14}C, or if any end-product is not readily prepared in a uniform layer for solid counting, it is usual to convert samples to CO_2 or $BaCO_3$ respectively. For this purpose, the wet-combustion technique (VAN SLYKE, PLAZIN and WEISIGER, 1952;

PETERS and GUTMAN, 1953; CALVIN *et al.*, 1949) has been successfully used (see also COLLINS and ROPP, 1955), although some exceptions have been reported. In the oxidation of [methyl-^{14}C] choline, a low radioactivity was observed in the resulting $BaCO_3$ which was shown to be due to a difference in the resistance to oxidation between the methyl and the ethanolamine moieties of choline (ARNSTEIN, 1952). With the wet combustion of iodoform, difficulties have been experienced (SHREEVE, LEAVER and SIEGEL, 1952), but other workers have found the method satisfactory (ROE and ALBENESIUS, 1952; ROPP, BONNER, CLARK and RAAEN, 1954). The former authors have devised a specific oxidation procedure for iodoform, and compared the results obtained with those found by standard wet combustion.

V. SOME APPLICATIONS OF ISOTOPES TO METABOLIC STUDIES

The main advantages of the isotopic tracer technique is its applicability, both *in vivo* and *in vitro*, to reaction sequences in which no net synthesis occurs. Thus, the incorporation of $^{14}CO_2$ by photosynthesis into intermediates has been followed under steady-state conditions (BENSON *et al.*, 1950). Reactions may be studied even during the simultaneous breakdown of tissue constituents, and the biosynthesis of purines from labelled glycine and formate in liver homogenates could be readily demonstrated despite the extensive catabolism of tissue nucleic acid to purines (GREENBERG, 1951). The utilization of precursors which are not rate limiting can also be shown without difficulty, as in the case of penicillin biosynthesis from cystine and valine (ARNSTEIN and GRANT, 1954a).

Moreover, isotopes may be used to assess the quantitative significance of one metabolic pathway with respect to other reactions of both the precursor and the product. This aspect has recently received increasing attention, particularly in cases where alternative pathways are possible.

1. *Choice of isotope*

The metabolic fate of an individual atom is usually best studied with an isotope of the same element. In early experiments, compounds were often labelled with deuterium or isotopic nitrogen, since isotopic carbon was not available. More recently, tritium has been used in a similar way (THOMPSON, 1954) because of its high specific activity and low cost. An important consideration governing the choice of tracers like deuterium and tritium for following the metabolism of carbon atoms to which the isotopic hydrogen is attached is the possibility of exchange of the isotope with cell water. Chemical stability can easily be checked, but metabolic reactions often give rise to intermediates in which the hydrogen atoms are labile. For example, succinate is converted into

acetate with the intermediate formation of oxaloacetate in which all hydrogen atoms are exchangeable with water. The labelled nitrogen atom of an amino acid may be similarly lost by transamination without metabolism of the carbon chain. No general rule can be laid down: some essential amino acids, like phenylalamine and tyrosine, undergo extensive transamination in the living animal, whilst others, such as threonine, are only irreversibly deaminated (ELLIOTT and NEUBERGER, 1950). On the other hand, glycine, though nutritionally dispensable and transaminated *in vitro* (CAMMARATA and COHEN, 1950), is not reversibly deaminated *in vivo*, as shown by a constant $^{14}C:^{15}N$ isotope ratio in glycine isolated from animals fed [α-^{14}C, ^{15}N]glycine (ARNSTEIN and NEUBERGER, 1951).

Care is therefore necessary in the choice of isotopes, but a study of the differences in the reactions of individual atoms or molecular fragments of a compound is, of course, essential for the complete understanding of its metabolism.

2. *Effect of dosage and diet*

Changes in the dietary level of a compound have been observed to alter its metabolism, but this factor is often not considered sufficiently. The oxidation of the methyl group of methionine by the rat is increased markedly when the level of [methyl-^{14}C] methionine in the diet is raised from 0·6 per cent to 1·2 per cent, the total amount of ^{14}C eliminated in the respiratory CO_2, faeces and urine within 24 hr being 10 per cent and 30 per cent respectively (MACKENZIE *et al.*, 1950). Moreover, the amount of [^{35}S] methionine incorporated into proteins is less with larger doses (TARVER, 1951), indicating that the proportions utilized for synthetic reactions (e.g. protein synthesis) or catabolized, depend on the amount given. Dietary choline also increased the oxidation of methionine methyl groups (MACKENZIE and DU VIGNEAUD, 1952), presumably because the methyl groups of cholines are transferred to methionine by transmethylation.

The metabolism of glycine is also strikingly affected by the dose and diet. With doses of 5 millimoles of [α-^{14}C] glycine/100 g body weight, extensive conversion of the α carbon atom of glycine into the β carbon atom of serine takes place *in vivo*, the radioactivity of the β carbon atom being 82 per cent of that of the α carbon atom of serine (SAKAMI, 1949). This reaction depends, however, on the level of glycine in the diet (Table 5). Since reduction in the dietary level of glycine does not increase the rate of glycine biosynthesis by the animal (ARNSTEIN and NEUBERGER, 1953b), the relative decrease in the radioactivity of the β carbon atom of serine must be due to a reduction in its biosynthesis from the α carbon atom of glycine. Since glycine is nutritionally dispensable and constitutes only about 0·2–0·5 per cent of normal diets,

TABLE 5

Effect of Dietary Glycine on the Biosynthesis of the β Carbon Atom of Serine from Glycine

Dietary level of [α-^{14}C] *glycine* (%)	*Distribution of radioactivity in serine isolated from tissue proteins* (%)*		
	β-C	α-C	*carboxy*-C
2	36	62	2
0·5	20	79	1
0·1	13	75	11

* ARNSTEIN and NEUBERGER (1953b); ARNSTEIN (unpublished results).

it is concluded that neither endogenously synthesized glycine nor glycine fed at normal nutritional levels is an important source of the one-carbon precursor of the β carbon atom of serine. The metabolic conversion of the α carbon atom of glycine, presumably to formaldehyde or formate, thus appears to be used mainly for the catabolism of excessive amounts of dietary glycine.

3. *Effect of radiation and the toxicity of isotopes*

A quantitative estimate of the radiation emitted by a radioactive compound used in a metabolic experiment is sometimes important, since the dose must not affect the metabolism of the experimental animal or tissue. On the basis of various assumptions concerning both the distribution of a particular isotope and the maximum permissible dose for prolonged exposure, the radiation received by various tissues has been calculated. Assuming a uniform distribution of isotope throughout the body, MARINELLI, QUIMBY and HINE (1948) have given the doses of several isotopes which emit radiation equivalent to 0·1 r during 24 hr after administration (see Table 6). It is clear, however, that uniform distribution is unlikely, an extreme example being the selective uptake of iodine by the thyroid gland. Recent recommendations by the International Commission on Radiological Protection (1954) have attempted to allow for uneven distribution of elements in the human body, and the radiation received by critical organs as well as by the whole body has been taken into account in assessing the maximum amount of a radioisotope which is considered safe to man for prolonged periods. The maximum dose is considered to be 0·3 rem/week radiation of 1 rem being defined as equivalent to X-radiation of such a dose that 1 r is absorbed by the body.

In biochemical experiments doses considerably in excess of those

TABLE 6

Dose of Isotope (μc/kg) giving a Radiation equivalent to (a) 0·1r for 24 hours after Administration or (b) 0·3r/week. Data for (a) calculated by MARINELLI *et al.* (1948), *assuming uniform Distribution of Isotope, those for (b) given in the report of the International Commission on Radiological Protection* (1954), *assuming non-uniform Distribution*

Isotope	*Dose (μc/kg)*	
	a	*b*
Tritium (^{3}H)	—	140
Carbon (^{14}C)	32	3
Phosphorus (^{32}P)	2·4	0·14
Sulphur (^{35}S)	30	4·3
Iodine (^{131}I)	8·3	0·08

in Table 6 (10–1000 times those in column *b*) are commonly used, but there is no evidence of harmful effects on the metabolism of the organism or tissue, which might invalidate the experimental results. Very high doses (up to 10^6 r) are required to cause physicochemical changes *in vitro*, such as depolymerization of nucleic acid, although doses as low as 50 r may produce chromosome damage. In general, animals are more susceptible than plants or micro-organisms, but species differences are relatively small, the LD_{50} ranging from 325 r for dogs to 800 r for rabbits (see review by ORD and STOCKEN, 1953). Most metabolic experiments are either of short duration or involve only very low doses. In addition, excretion of the administered compound is usually rapid. The amount of radiation in such experiments is therefore usually well below 50 r. In doubtful cases, the effect of radiation on the experimental results can be checked easily by repeating the experiment with identical doses containing different levels of radioactivity.

With deuterium, care must be taken to use safe isotope concentrations. Up to 10 per cent D_2O had no detectable effect on mammalian tissue (SCHOENHEIMER and RITTENBERG, 1940), and up to 50 per cent had no effect on the growth of algae (WEINBERGER and PORTER, 1954).

USE OF UNIFORMLY LABELLED COMPOUNDS

Uniformly labelled compounds, such as ^{14}C and ^{15}N amino acids, ^{14}C sugars, and ^{15}N and ^{32}P nucleotides, are useful mainly for general surveys of metabolic reactions. For example, the nitrogen metabolism of 15 biosynthetically prepared [^{15}N]amino acids has been investigated by ÅQVIST (1951). Similarly, the conversion of carbohydrate into various amino acids was demonstrated with uniformly labelled [^{14}C] glucose both *in vitro* (WINZLER *et al.*, 1952) and *in vivo* (BLACK, KLEIBER and BAXTER, 1955), and with [^{14}C] sucrose (STEELE, 1952).

In vivo, carbohydrate was converted only into the nonessential amino acids, but *in vitro* brain tissue incorporated radioactivity into all amino acids except proline and threonine, whereas liver and intestine gave similar results to the *in vivo* experiment (WINZLER *et al.*, 1952).

1. *Isotopic competition*

In this method, the effect of unlabelled compounds on the incorporation of isotope from a labelled substrate into a metabolic product is determined, since the addition of intermediates or closely related compounds results in a decreased utilization of the labelled substrate. This method is often very convenient, because the preparation of labelled intermediates is avoided, whilst the labelled substrate is usually a readily accessible compound such as $^{14}CO_2$, uniformly labelled glucose, or even an inorganic ion (e.g. $^{35}SO_4^{=}$, $^{32}PO_4^{\equiv}$). Thus, the utilization of L-cystine, L-cysteine, methionine, or glutathione for the biosynthesis of penicillin by *Penicillium chrysogenum* was shown by their effect on the incorporation of ^{35}S from labelled inorganic sulphate (STEVENS *et al.*, 1953). Other sulphur compounds, including DL-homocysteine, L-cysteic acid, and taurine, were also converted into penicillin in preference to sulphate, but less efficiently than L-cystine (STEVENS *et al.*, 1954). Similarly, the utilization of labelled sulphate by *Escherichia coli* in the presence and absence of various organic sulphur compounds indicates the following metabolic pathway for the conversion of inorganic to organic sulphur by *E. coli* (COWIE, BOLTON and SANDS, 1951):

$$SO_4^{=} \rightarrow \begin{cases} SO_3^{=} \\ S_2O_3^{=} \end{cases} \rightarrow \text{cysteine} \rightarrow \text{homocysteine} \rightarrow \text{methionine}$$

Amino-acid metabolism has also been studied by the isotopic competition method, using uniformly labelled [^{14}C] glucose, $^{14}CO_2$ or [^{14}C] acetate, as the labelled substrate (ROBERTS *et al.*, 1953).

2. *Trapping of intermediates*

Since intermediates are often synthesized in trace amounts only, metabolic reactions are sometimes studied in the presence of a large excess of an unlabelled compound which is believed to be an intermediate. The incorporation of label into the added compound shows that this intermediate occurs in the metabolic sequence being investigated. The absence of incorporation of isotope, however, is not conclusive evidence. For example, when labelled acetate is oxidized by *Escherichia coli* in the presence of unlabelled α ketoglutarate and succinate, little radioactivity was incorporated into α ketogluturate, but the succinate was highly labelled. On the other hand, in the absence

of unlabelled α ketoglutarate, the α ketoglutarate produced metabolically was labelled to the same extent as the other acids of the tricarboxylic acid cycle, indicating that exogenous α ketoglutarate does not equilibrate with metabolic α ketoglutarate (SWIM and KRAMPITZ, 1954; AJL and WONG, 1955).

USE OF SPECIFICALLY LABELLED COMPOUNDS

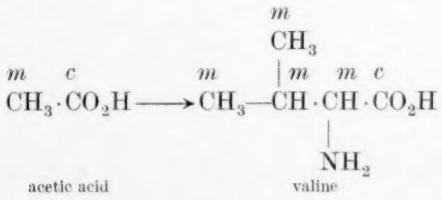
1. *Labelling of specific positions with one isotope*

The selective labelling of a precursor in a known position may be used to elucidate reaction mechanisms. Thus, the distribution of ^{14}C in glycine derived from labelled acetate excluded the possibility of a direct conversion of acetate into glycine, since carboxyl-labelled acetate gave rise to glycine labelled only in the carboxyl carbon atom, whilst [α-^{14}C] acetate was converted into glycine containing 60–70 per cent of the total ^{14}C in the α carbon atom and 30–40 per cent in the carboxyl carbon atom (ARNSTEIN and NEUBERGER, 1949).

In many cases it is, however, necessary to consider the possibility of molecular rearrangements and migrations which may result in an unexpected distribution of isotope in the metabolic product. In addition, asymmetric labelling does not necessarily exclude a symmetrical intermediate, as will be discussed in detail later.

The occurrence of interesting intramolecular rearrangements in the biosynthesis of valine, isoleucine and cholesterol has been deduced recently from the distribution of isotope when these compounds were biosynthesized from precursors labelled in known positions.

Valine and isoleucine. In the biosynthesis of valine from acetate by *Torulopsis utilis* only the carboxyl carbon atom of valine is derived from the carboxyl carbon atom (*c*), whilst all the other carbon atoms originate from the methyl carbon atom (*m*) of acetate (EHRENSVÄRD *et al.*, 1951):

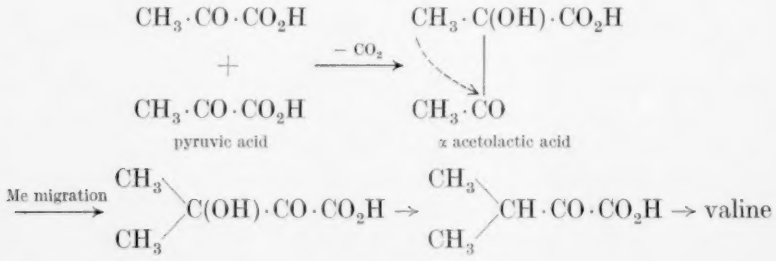


This distribution of isotope eliminated the possibility that the carbon skeleton of valine might be synthesized from acetate *via* acetoacetate and ββ dimethylacrylic acid, which would result in labelling of the β carbon atom of valine from [carboxy-^{14}C]acetate. A comparison of the distribution of ^{14}C in valine derived from labelled acetate, lactate

and glucose (STRASSMAN, THOMAS and WEINHOUSE, 1955), showed that [2-^{14}C] lactate gave rise to equal labelling of the α and β carbon atoms, whereas only the carboxyl carbon atom was labelled when [1-^{14}C] lactate was the precursor. [1-^{14}C] Glucose gave rise to valine labelled predominantly in the methyl carbon atoms, presumably after conversion into [3-^{14}C] lactate:

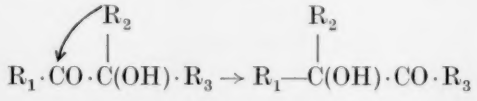
Precursor	Radioactivity in valine			
	γ CH_3	β	α	
		$CH \cdot CH(NH_2) \cdot CO_2H$		
	γ' CH_3			
$CH_3 \cdot {}^{14}CHOH \cdot CO_2H$	20	700	750	45
$CH_3 \cdot CHOH \cdot {}^{14}CO_2H$	—	—	—	905
[1-^{14}C] glucose	358	50	45	25

This distribution of isotopic tracer can be explained satisfactorily by a mechanism involving the initial condensation of two molecules of pyruvate, derived from lactate, to α acetolactate followed by migration of a methyl group:



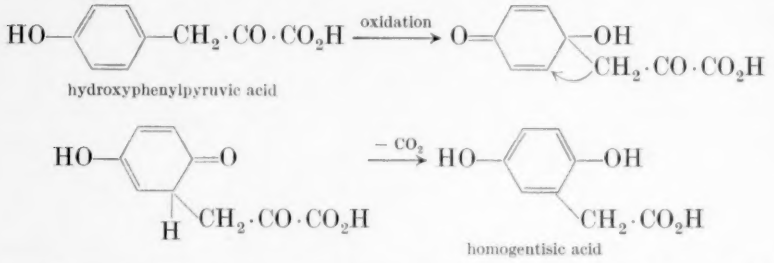
The widespread distribution of such a pathway of valine biosynthesis among micro-organisms is suggested by the similar results obtained recently with *Aerobacter aerogenes* (RAFELSON, 1955) and *Saccharomyces cerevisiae* (MCMANUS, 1954).

In discussing this proposed mechanism, STRASSMAN *et al.* (1955) drew attention to the possibility that such reactions, resembling the pinacol rearrangement of ditertiary glycols,

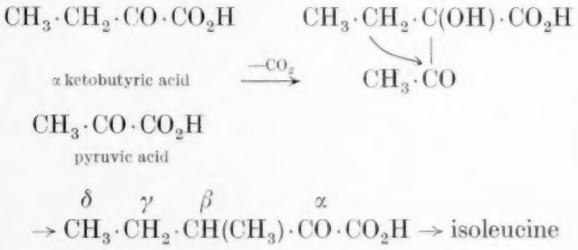


may be of more general occurrence. Thus, *p*-hydroxyphenylpyruvic acid may be converted into homogentisic acid by oxidation and

rearrangement of the intermediate, which is a vinylog of an $\alpha\beta$ hydroxyketone:

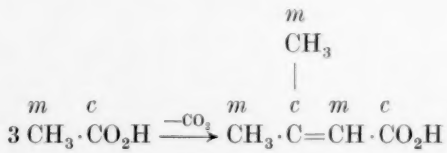


The biosynthesis of isoleucine by *Torulopsis utilis* also takes place by a similar mechanism, pyruvate (or acetaldehyde derived from pyruvate) condensing with α ketobutyrate, instead of with a second molecule of pyruvate (STRASSMAN *et al.*, 1954):

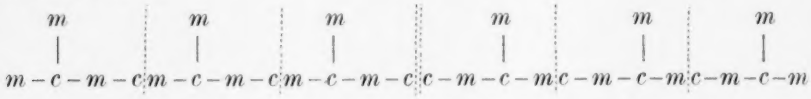


The relationship of threonine to isoleucine biosynthesis has been clarified by isotope-competition experiments using *Neurospora crassa* (ADELBERG, 1955). The incorporation of radioactivity from [1, 2-$^{14}C_2$] acetate into the carboxyl, α, γ and δ atoms of the isoleucine precursor was suppressed by the addition of unlabelled L-threonine. Moreover, [*carboxy*, α-$^{14}C_2$] threonine labelled the carboxyl and α carbon atoms of this precursor, indicating that threonine is converted into α ketobutyrate or a closely related 4-carbon compound.

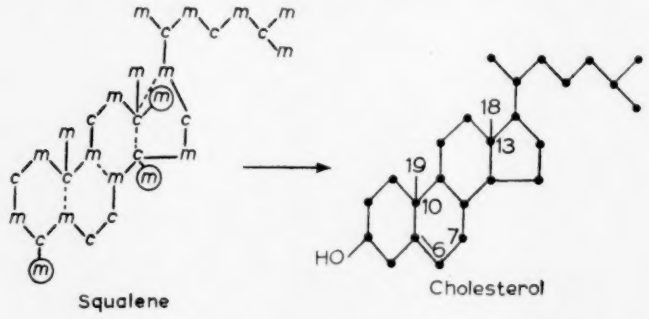
Cholesterol. An intramolecular migration of a methyl group also occurs in the biosynthesis of cholesterol. As discussed in an excellent review by CORNFORTH (1954), acetate is first converted into a C_5 precursor, which may be a compound closely related to $\beta\beta$ dimethylacrylic acid (BLOCH, CLARKE and HARARY, 1955):



This C_5 precursor is converted into squalene, which then condenses to cholesterol. The conversion of squalene into cholesterol and its formation from acetate has been established by tracer experiments (LANGDON and BLOCH, 1953a,b; POPJÁK, 1954) and the labelling pattern in squalene agrees with that predicted by the condensation of six C_5 units, where *m* and *c* denote carbon atoms derived from the methyl and carboxyl groups of acetate, respectively (CORNFORTH and POPJÁK, 1954):

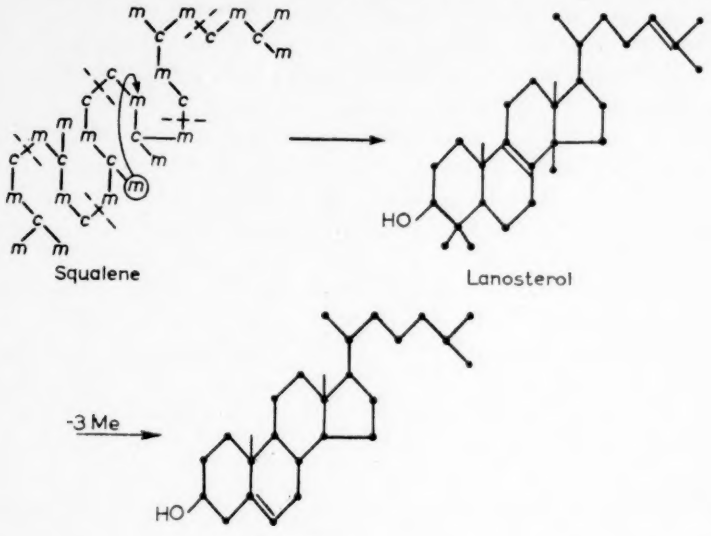


According to the old squalene hypothesis (ROBINSON, 1934), the distribution of labelled atoms in cholesterol formed from squalene should be as follows, three methyl groups (ⓜ) being lost during the cyclization:



In particular, C_6, C_7, C_{10} and C_{13} should be derived from carboxyl carbon atoms of acetate, and C_{18} and C_{19} from methyl carbon atoms. Experiments to test this hypothesis showed, however, that both C_7 (BLOCH, 1953) and C_{13} (WOODWARD and BLOCH, 1953) are derived from methyl carbon atoms of acetate. WOODWARD and BLOCH (1953) therefore suggested a different cyclization of squalene involving the migration of one methyl group (ⓜ) to give lanosterol, followed by conversion into cholesterol with loss of three methyl groups. (See illustration at top of next page.)

This mechanism also accounts satisfactorily for the biosynthesis of lanosterol, which is not constituted in accordance with the isoprene rule. The mechanism of the methyl group migration is not known, but evidence for the metabolic pathway squalene → lanosterol → cholesterol has been reported recently (TCHEN and BLOCH, 1955).



Porphyrins. In the case of the porphyrins, the distribution of isotope in the pyrrole rings and methyne bridges (WITTENBERG and SHEMIN, 1950) suggests the intramolecular migration of a one-carbon fragment. Alternative mechanisms (BOGORAD and GRANICK, 1953; SHEMIN, RUSSELL and ABRAMSKY, 1955) could, however, equally well account for the experimental findings.

2. *Asymmetrical labelling in the biosynthesis of metabolic products*

Contrary to earlier views, it is now well established that the asymmetrical labelling of a metabolic product does not exclude the participation of symmetrical intermediates in its biosynthesis. As pointed out by OGSTON (1948), a symmetrical compound may be metabolized like an asymmetrical one, provided that a three-point combination between substrate and enzyme occurs and reaction is only possible at

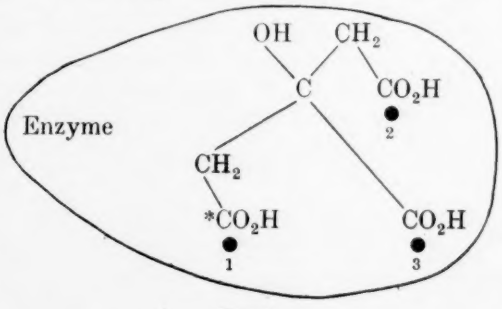


Fig. 2.

one of the points. If, for example, all three carboxyl groups of citric acid have to be attached to points 1, 2 and 3 on the enzyme (Fig. 2), there is only one possible arrangement and the carboxyl group marked with an asterisk is always at point 1.

Citric acid. Direct proof that citric acid behaves metabolically like an unsymmetrical molecule was obtained by POTTER and HEIDELBERGER (1949), LORBER, UTTER, RUDNEY and COOK (1950), and MARTIUS and SCHORRE (1950). Radioactive citric acid was synthesized by a rat-liver homogenate in the presence of $^{14}CO_2$ and, after its isolation, the labelled citrate was converted into α ketoglutarate in a second liver homogenate (POTTER and HEIDELBERGER, 1949; LORBER *et al.*, 1950). Only the α carboxyl carbon atom of α ketoglutarate was labelled, proving that citric acid was both synthesized and metabolized asymmetrically (see Fig. 3), since symmetrical metabolism would give equal radioactivities in both carboxyl groups of α ketoglutarate.

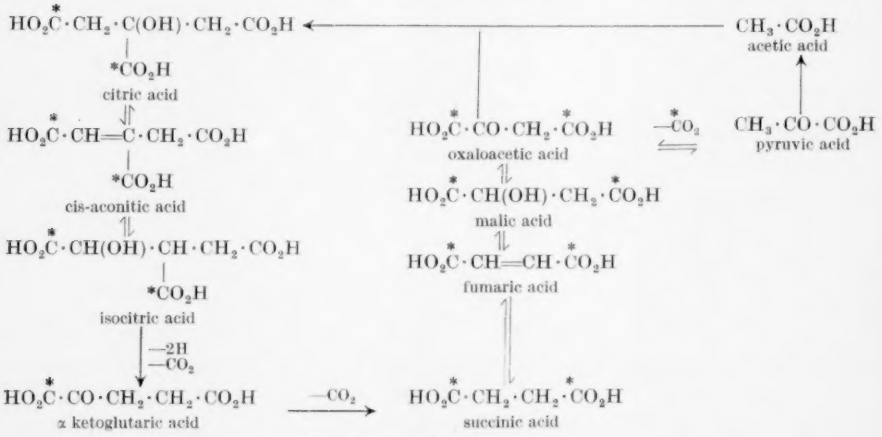
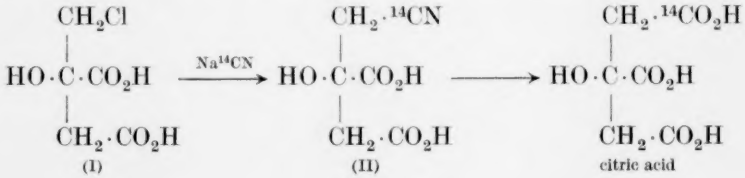
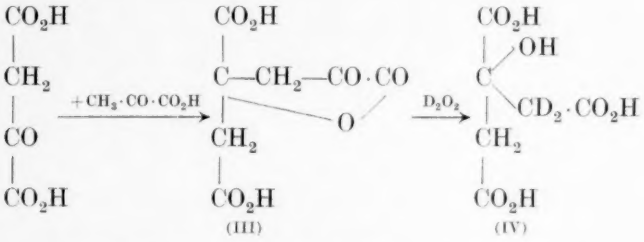


Fig. 3. The citric acid cycle.

Additional evidence was obtained by WILCOX, HEIDELBERGER and POTTER (1950) with synthetic citric acid labelled with ^{14}C in only one of the primary carboxyl groups. The labelled citric acid was synthesized by condensing $Na^{14}CN$ with optically active β-carboxy-γ-chloro-γ-hydroxybutyric acid (I), followed by hydrolysis of the nitrile. Labelled citric acid prepared from the laevorotatory chlorocompound (I)

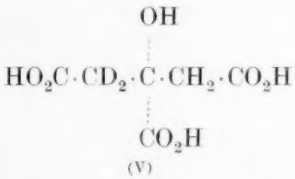


was metabolized to α ketoglutarate labelled only in the γ carboxyl carbon atom. Essentially similar results were obtained with deuterium-labelled citric acid (IV) (MARTIUS and SCHORRE, 1950), which was synthesized from both stereoisomers of oxalocitramalic acid lactone (III):



In this case, the labelled citric acid (IV) actually had a measurable rotation. The laevorotatory form was converted by pigeon breast muscle into α ketoglutarate having the same deuterium content as the starting material, but the dextrorotatory enantiomorph lost all the deuterium since this position becomes the keto group of α ketoglutarate.

It is moreover possible to correlate the metabolic origin with the stereochemical structure of citric acid. If the formula (IV) is written according to the Fischer convention with the hydroxyl and tertiary carboxyl groups behind the plane of the paper, the form derived from the laevorotatory lactone (III) contains deuterium in the left-hand CH_2 group (V):



It is this form which is converted into α ketoglutarate labelled with deuterium, and since it is known from other isotope experiments that the C_2 precursor ("active" acetate) is recovered in α ketoglutarate, the right-hand—$CH_2 \cdot CO_2H$ group must be derived from oxaloacetate and the left-hand one from the C_2 unit condensing with it.

The type of compound which can behave asymmetrically in enzymic reactions or, more generally, with asymmetric reagents, has been defined by RACUSEN and ARONOFF (1951), who suggested that discrimination between identical groups is possible only in the case of molecules which do not possess two or more axes of symmetry. SCHWARTZ and CARTER (1954) have pointed out, however, that any "*meso* carbon atom," i.e. a carbon atom carrying two identical (*a*,*a*) and two different (*b*,*d*) substituents (Fig. 4, I) would be expected to yield unequal

amounts of diastereoisomers by reacting with an appropriate asymmetric reagent. This definition includes a compound containing two identical meso carbon atoms and thus two axes of symmetry (Fig. 4, II).

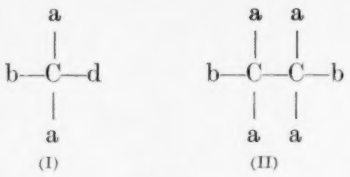
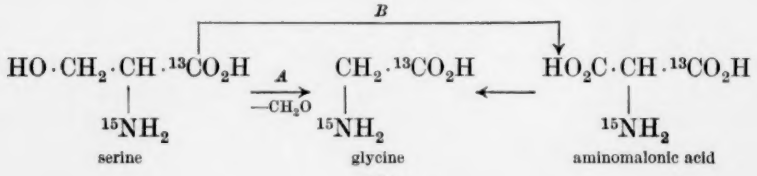


Fig. 4.

Glycerol. Like citric acid, glycerol is metabolized asymmetrically. Biosynthetic [1-^{14}C] glycerol, obtained either from the milk fat of a goat which had been injected with [1-^{14}C] acetate (SCHAMBYE, WOOD and POPJÁK, 1954) or by fermentation of [3:4-$^{14}C_2$] glucose by yeast (SWICK and NAKAO, 1954), was metabolized by rats to glucose in which 90 per cent of the total radioactivity was located in carbon atoms 3 and 4. Chemically synthesized [1-^{14}C] glycerol, on the other hand, was converted into glucose labelled equally in C_1, C_3, C_4 and C_6 (DOERSCHUK, 1952), or into lactate labelled in $C_{(1)}$ and $C_{(3)}$ (TENG *et al.*, 1953). The latter findings are not surprising since the chemical reactions used in this synthesis would give rise to glycerol labelled equally in both $\cdot CH_2OH$ groups.

The fermentation of [3:4-$^{14}C_2$] glucose by *Saccharomyces chevalieri* gives L-α-glycerophosphate containing 83 per cent of the total radioactivity at C_3, i.e. the unphosphorylated primary carbinol carbon atom (BUBLITZ and KENNEDY, 1954) and biosynthetic glycerol, arising by the reaction sequence glucose → hexose 1:6-diphosphate → dihydroxyacetone 1-phosphate → L-α-glycerophosphate → glycerol, would therefore be labelled almost entirely in only one of two $\cdot CH_2OH$ groups. Reversal of this pathway accounts satisfactorily for the asymmetric metabolism of glycerol, since the phosphorylation of glycerol is stereochemically specific, giving only L-α-glycerophosphate (KALCKAR, 1939).

Serine. Serine labelled with ^{13}C in the carboxyl carbon atom and with ^{15}N is metabolized to glycine which contains both isotopes in the same ratio as the precursor (SHEMIN, 1946). This observation was believed to exclude aminomalonic acid (pathway B) as an intermediate



in this reaction. OGSTON (1948) pointed out, however, that the unchanged isotope ratio could also be explained if aminomalonic acid, like citric acid, could give rise to an unsymmetrical enzyme-substrate complex in which only one of the two carboxyl groups, i.e. that derived from the hydroxymethyl group of serine, might be lost as CO_2. As a result of further work, however, it is now clear that in the serine-glycine reaction the hydroxymethyl group of serine is converted into a derivative of formaldehyde (ELWYN, WEISSBACH, HENRY and SPRINSON, 1955) and pteroylglutamic acid (BLAKLEY, 1954) (pathway *A*), and aminomalonic acid is therefore not an intermediate.

3. *Studies of reaction mechanisms by mass analysis of product*

In two cases, analysis of the mass of a metabolic product synthesized from a precursor containing a high concentration of ^{13}C has been used to study the biosynthetic mechanism.

The observation that acetate synthesized from glucose by *Chlostridium thermoaceticum* is labelled in both the methyl and carboxyl carbon atoms when $^{14}CO_2$ is present during the fermentation suggested the following reactions (BARKER and KAMEN, 1945):

$$\left.\begin{aligned} C_6H_{12}O_6 + 2H_2O &\rightarrow 2CH_3\cdot CO_2H + 8\,H + 2\,CO_2 \\ 2^{14}CO_2 + 8H &\rightarrow {}^{14}CH_3\cdot{}^{14}CO_2H + 2H_2O \end{aligned}\right\}$$

Chemical degradation cannot distinguish, however, between doubly-labelled acetate, $^*CH_3\cdot{}^*CO_2H$, and a mixture of singly-labelled acetates, $^*CH_3\cdot CO_2H + CH_3\cdot{}^*CO_2H$. Moreover, ^{14}C is available only in relatively low isotope concentration, and the condensation of two molecules of labelled CO_2 (i.e. $^{14}CO_2$ + large excess $^{12}CO_2$) would therefore give mainly $^{12}CH_3\cdot{}^{12}CO_2H$ together with a mixture of $^{14}CH^3\cdot{}^{12}CO_2H + {}^{12}CH_3\cdot{}^{14}CO_2H$ + only a few doubly labelled molecules, $^{14}CH^3\cdot{}^{14}CO_2H$. Both difficulties were overcome by using a high concentration of $^{13}CO_2$ as tracer and determining the absolute mass of the biosynthetic acetic acid (WOOD, 1952). The acetic acid was converted into ethylene, the relative abundance of $H_2{}^{13}C{:}{}^{13}CH_2$ (mass 30), $H_2{}^{13}C{:}{}^{12}CH_2$ (mass 29) and $H_2{}^{12}C{:}{}^{12}CH_2$ (mass 28) being measured directly in a mass spectrometer. All three types of molecules were present in amounts corresponding to 30 per cent $^{13}CH_3\cdot{}^{13}CO_2H$, 30 per cent $^{12}CH_3\cdot{}^{13}CO_2H$ and 40 per cent $^{12}CH_3\cdot{}^{12}CO_2H$. It is, however, not necessary to assume the direct condensation of two molecules of $^{13}CO_2$ to form $^{13}CH_3{}^{13}\cdot CO_2H$, since the above results can also be explained by the reaction of a labelled C_1 compound with a singly labelled C_2 unit, followed by loss of the unlabelled carbon atom, viz.

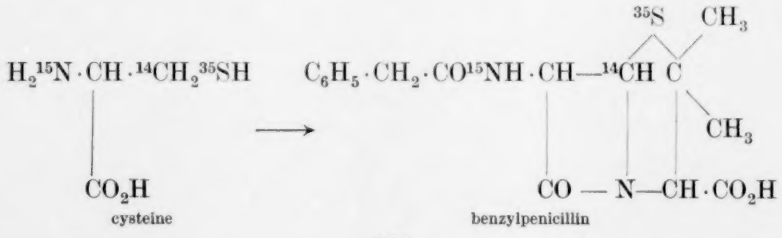
$$^{13}C + {}^{13}C\cdot{}^{12}C \rightarrow {}^{13}C\cdot{}^{13}C\cdot{}^{12}C \rightarrow {}^{13}C\cdot{}^{13}C + {}^{12}C$$

Direct mass analysis has been used with more conclusive results to distinguish between the biosynthesis of succinate by the direct condensation of two molecules of acetate (Thunberg condensation) and its biosynthesis by the tricarboxylic acid cycle (see Fig. 3). These mechanisms would convert $^{13}CH_3 \cdot CO_2H$ into $HO_2C \cdot {}^{13}CH_2 \cdot {}^{13}CH_2 \cdot CO_2H$ and $HO_2C \cdot {}^{13}CH_2 \cdot {}^{12}CH_2 \cdot CO_2H$, respectively. Mass analysis of the ethylene obtained from the α and β carbon atoms of succinate showed that none of the succinate molecules were doubly labelled, thus excluding the direct (Thunberg) condensation (SWIM and KRAMPITZ, 1954b).

4. *Multiple labelling with two or more isotopes*

The utilization of a particular compound as such or of a specific part of it as a precursor without prior extensive chemical modification can often be demonstrated conveniently by labelling with two or more isotopes. The incorporation of both, or all, isotopes into the metabolic product without alteration in their relative proportion then indicates the retention of chemical bonds between the labelled positions of the precursor. A change in the isotope ratio on the other hand shows that degradation of the precursor into smaller fragments must have occurred.

Early applications of double labelling include studies of the conversion of methionine, labelled with ^{34}S and ^{13}C, into cystine (DU VIGNEAUD, KILMER, RACHELE and COHN, 1944), transmethylation reactions of the methyl group of methionine containing ^{14}C and deuterium (KELLER, RACHELE and DU VIGNEAUD, 1949), the conversion of serine into glycine (SHEMIN, 1946), the acetylation of foreign amines (BLOCH and RITTENBERG, 1945), and the biosynthesis of porphyrins (WITTENBERG and SHEMIN, 1950; MUIR and NEUBERGER, 1950). More recently, the biosynthesis of penicillin has been investigated with cystine labelled with ^{14}C, ^{35}S and ^{15}N (ARNSTEIN and GRANT, 1954b). Both the position of the isotope in the penicillin molecule and the identical ratios of the three isotopes in the labelled precursor, in penicillin and in cystine isolated from the mycelial protein, indicated the direct conversion of cysteine into penicillin, as follows:



The possibility that phenylacetylcysteine might be an intermediate in the synthesis of benzylpenicillin was tested with diphenylacetylcystine (I) labelled with ^{14}C and ^{35}S.

$$[C_6H_5 \cdot CH_2 \cdot {}^{14}CONH \cdot CH \cdot CH_2{}^{35}S]_2$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad CO_2H$$

(I)

In this case, however, extensive breakdown and resynthesis of the labelled precursor, as shown by a change in the $^{14}C:{}^{35}S$ ratio (7·3 at the beginning and 0·81 at the end of the penicillin-producing fermentation), did not permit an unequivocal interpretation of the experimental results (Arnstein, Clubb and Grant, 1954).

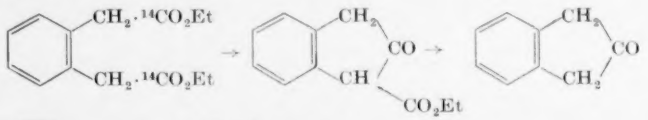
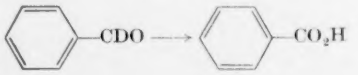
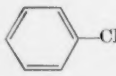
5. *Isotope effects and the interpretation of tracer experiments*

Since isotopes differ in mass and zero-point energy, quantitative comparisons of the utilization of different isotopes for metabolic reactions are always subject to possible errors due to differences in reaction rates. In all chemical reactions which have been investigated so far, molecules containing a heavy isotope have been found to react more slowly, as shown by some recent examples given in Table 7. Differences in reaction rates between ^{14}C, ^{13}C and ^{12}C are usually about 5–10 per cent, whilst those between ^{15}N and ^{14}N are smaller. On the other hand much larger isotope effects have been observed with the isotopes of hydrogen. (For recent reviews see Ropp, 1952; Yankwich, 1953 and Eidinoff, 1953.)

The effect of isotopes on biological processes has been of interest ever since the discovery of deuterium. At first mainly the gross physiological effects of heavy water were studied (Schoenheimer and Rittenberg, 1940), but in a few experiments the rates of reactions involving C—H and C—D bonds were determined. Thus, the rate of dehydrogenation of normal succinic acid by succinic dehydrogenase was 30 per cent faster than that of succinic acid containing one atom of deuterium in each of the methylene groups (Erlenmeyer, Schoenauer and Süllman, 1936), whilst the difference in reaction rates of normal and tetradeuterio-succinic acid was as high as 80 per cent (Sonderhoff and Thomas, 1937). More recently, detailed kinetic studies of the dehydrogenation of deuterium-labelled succinic acid confirmed that normal succinate is oxidized faster, and showed that the affinity of the dehydrogenase for the unlabelled compound was about 1·45 times that for succinic acid containing 77 atom per cent D in the methylene groups (Thorn, 1951). Similar differences have been observed in several other processes. When *Chlorella pyrenoidosa* was grown in labelled water, tritium and deuterium were incorporated into the cells

TABLE 7

Isotope Effects in some Chemical Reactions. The Isotope Effect is given by the Ratio of the Velocity Constants for Reactions of the Labelled and Unlabelled Compounds, where the Subscripts refer to ^{14}C, ^{13}C, ^{13}C, *Deuterium and Hydrogen*

Reaction	*Isotope effect*	*Reference*
$2HCO_2Hg \rightarrow CO_2 + 2Hg + HCO_2H$	$k_{14} < k_{12}$	Ropp, Raaen, and Weinberger (1953)
Acetophonone → 2:4-dinitrophenylhydrazone derivative	$k_{13}/k_{12} = 0{\cdot}946$	
Benzophenone → 2:4-dinitrophenylhydrazone derivative	$k_{14}/k_{12} = 0{\cdot}936$	
Urea $\xrightarrow{H^+}$ CO_2	$k_{13}/k_{12} = 0{\cdot}94$	Schmitt and Daniels (1953)
	$k_{14}/k_{12} = 0{\cdot}9$	
$HO_2C{\cdot}CH_2{\cdot}\overset{*}{C}O_2H \rightarrow CH_3{\cdot}CO_2H + CO_2$	$k_{13}/k_{12} = 0{\cdot}97$	Yankwich and Stevens (1953)
	$k_{14}/k_{12} = 0{\cdot}91$	
$HO_2C{\cdot}CHBr{\cdot}\overset{*}{C}O_2H \rightarrow CH_2Br{\cdot}CO_2H + CO_2$	$k_{13}/k_{12} = 0{\cdot}98$	
	$k_{14}/k_{12} = 0{\cdot}90$	
$CH_3{\cdot}CO{\cdot}{}^{14}CH_3 \rightarrow CH_3{\cdot}NH_2 + CH_3{\cdot}CO_2H$	$k_{14} < k_{12}$	Ropp, Bonner, Clark and Raaen (1955)
	$k_{14}/k_{12} = 0{\cdot}95$ (intramolecular)	Carrick and Fry (1955)
	$k_{14}/k_{12} = 0{\cdot}92$ (intermolecular)	
	$k_D/k_H = 0{\cdot}23$	Wiberg (1954)
 CDO Cannizaro reaction	$k_D/k_H = 0{\cdot}55$	

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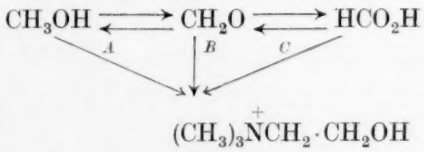
at 50 per cent and 55 per cent of the rate of hydrogen, respectively (WEINBERGER and PORTER, 1954). The incorporation of tritium relative to deuterium from heavy water into the fatty acids of mammary gland and liver fat is about 0·775 and 0·87, respectively (GLASCOCK and DUNCOMBE, 1954), but less fractionation of hydrogen isotopes was observed in liver glycogen (EIDINOFF *et al.*, 1953). Considerably smaller differences than the above for the relative incorporation of deuterium and tritium into fat have also been reported (THOMPSON and BALLOU, 1954), possibly due to the longer experimental period used.

GLASCOCK and DUNCOMBE (1954) have suggested that, in the synthesis of a metabolite by reversible reactions, isotope fractionation will be observed only under conditions of net synthesis but not under steady-state conditions. It should be emphasized that these considerations apply only to a metabolic product which is degraded by reversal of the reactions by which it is synthesized, as is the case with fatty acids (LYNEN and OCHOA, 1953). When synthesis and breakdown take place by different reactions, biological fractionation of isotopes may occur also in the absence of net synthesis.

Quantitatively important fractionation occurs mainly with the isotopes of hydrogen, since differences in their mass are much larger than those of the isotopes of carbon, nitrogen, oxygen and the heavier elements. Recent work suggests, however, that in some cases differences in the utilization of ^{14}C and ^{12}C may approach those of the hydrogen isotopes, possibly due to cumulative isotope effects in a series of reactions. Thus, $^{14}CO_2$ and $^{13}CO_2$ are used by photosynthesizing *Chlorella* cells or barley leaves approximately 15 per cent and 4 per cent, respectively, more slowly than $^{12}CO_2$ (VAN NORMAN and BROWN, 1952; WEIGL and CALVIN, 1949).

Methyl group biosynthesis. Fractionation of hydrogen isotopes is particularly important when double labelling is used to study reactions involving oxidation-reduction mechanisms. As an example, the conversion of methanol and related compounds into methyl groups, which has recently been studied by DU VIGNEAUD and his collaborators, will be discussed in some detail. Methanol, formaldehyde and formate, labelled with ^{14}C, had all been shown to serve as precursors of the methyl group of choline (DU VIGNEAUD *et al.*, 1951) and it was of interest, therefore, to distinguish between the following possible alternative pathways *A*, *B* and *C*:



In the conversion of a mixture of $^{14}CH_3OH$ and CD_3OH into the methyl group of choline, only about 25–30 per cent of the deuterium accompanied the isotopic carbon, indicating that not all of the methanol was used directly (pathway *A*). A comparison of the utilization of $CH_2TOH + {}^{14}CH_3OH$ and $CD_3OH + {}^{14}CH_3OH$ showed, however, that the loss of deuterium relative to that of labelled carbon was about three times that of tritium; i.e. approximately 75 per cent of the tritium was retained (VERLY *et al.*, 1952). In more strictly comparable experiments with labelled methanol consisting of a mixture of CH_2DOH (instead of CD_3OH), CH_2TOH and $^{14}CH_3OH$, the retention of tritium relative to deuterium was much reduced, the methyl groups of choline and creatine containing about 1·3 times more tritium relative to ^{14}C than deuterium (RACHELE, KUCHINSKAS, KNOLL and EIDINOFF, 1954). The isotope content of formate in this experiment showed that in the oxidation of methanol to formate 1·7 times more deuterium is lost than tritium. These results are summarized in Table 8, where F_T and F_D

TABLE 8

Isotope Effects in the Utilization of Labelled Methanol for Methyl Group and Formate Biosynthesis. F_T and F_D are factors giving the retention of Tritium and Deuterium, respectively, relative to ^{14}C (VERLY *et al.*, 1952; RACHELE *et al.*, 1954). *F_H* is the Retention Factor for Isotopic Hydrogen which has been corrected for Isotope Effects (see text).*

Expt.	*Precursor injected*	*Metabolic product*	*Relative utilization of hydrogen isotopes*			
			F_T	F_D	F_T/F_D	F_H*
A	$^{14}CH_3OH$	Choline methyl group	0·75	0·22	3·4	
			1·02	0·36	2·9	
	CD_3OH	Creatine methyl group	0·69	0·22	3·1	
	CH_2TOH		0·86	0·28	3·1	
B	$^{14}CH_3OH$	Choline methyl group	0·804	0·640	1·26	0·57
			0·875	0·690	1·27	0·61
	CH_2DOH	Creatine methyl group	0·581	0·439	1·32	0·38
	CH_2TOH	Formyl group of formate	1·98	1·16	1·72	0·82

give the retention of tritium and deuterium, respectively, relative to isotopic carbon. These factors are obtained by dividing the isotope ratio $T/^{14}C$ or $D/^{14}C$ of the methyl group of the product by that of the methanol. The ratio F_T/F_D then indicates the preferential utilization of tritium relative to deuterium. These differences can be explained by the preferential rupture of C—H bonds during the oxidation of methanol, which results in the formation of intermediates enriched in deuterium

or tritium when CH_2TOH or CH_2DOH are used. As expected from the usual isotope effect, C—H, C—D, and C—T bonds react at decreasing rates in agreement with both chemical and biochemical experiments. Thus, the oxidation of deuterium-labelled benzaldehyde ($C_6H_5 \cdot CDO$) (Wiberg, 1954) and acetaldehyde ($CH_3 \cdot CDO$) (Cornforth and Popják, 1949) is slower than that of the unlabelled compounds, and the *in vivo* oxidation of the methyl group of methionine labelled with deuterium and ^{14}C (i.e. $^{14}CD_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$) is only 60–80 per cent as fast as the oxidation of that containing only hydrogen and ^{14}C($^{14}CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$) (Rachele, Kuchinskas, Kratzer and du Vigneaud, 1955). On the other hand, the oxidation of CD_3OH does not give rise to intermediates of enriched isotope content, but with a mixture of CD_3OH and $^{14}CH_3OH$ preferential reaction of the latter would still occur, resulting in a relatively lower incorporation of deuterium into oxidation products. This is shown by the lower incorporation of deuterium relative to ^{14}C(F_D) from a mixture of $CD_3OH + {}^{14}CH_3OH$ compared with that from $CH_2DOH + {}^{14}CH_3OH$ (experiments *A* and *B*, Table 8).

The interpretation of these experiments is thus complicated by the magnitude of the observed isotope effects. As has been pointed out (Rachele *et al.*, 1955), possible ambiguities due to isotope effects may be minimized by "intramolecular" double labelling. Relatively high isotope concentrations are essential for such experiments, and the only suitable isotopes are, therefore, deuterium and ^{13}C. In the example discussed here, the metabolism of labelled methanol in which deuterium is attached only to isotopic carbon, i.e. $^{13}CD_3OH$, should indicate more conclusively the oxidation level of intermediates. Alternatively, the experimental results obtained with methanol labelled identically with either deuterium or tritium (i.e. CH_2DOH and CH_2TOH) may be corrected for isotope effects, on the assumption that differences in reaction rates are due solely to differences in the zero-point vibration energy of C—H, C—D and C—T bonds. On this basis, the rate constants of reactions involving C—H bonds are approximately 6·3 and 16·3 times those involving C—D and C—T bonds, respectively (Eidinoff, 1953). In this way, corrected retention factors for isotopic hydrogen relative to carbon have been calculated (F_H*, Table 8). It will be seen that this correction is at best only approximate, since in the case of formate F_H* should be 1. The results suggest, however, that in the conversion of methanol to choline methyl groups oxidation to the level of formaldehyde takes place prior to reduction (pathway *B*, p. 199). The better retention of isotopic hydrogen during the biosynthesis of choline methyl groups compared with that of creatine is also of interest as indicative of a different reaction mechanism.

Similar isotope effects occur in the metabolic reactions of several

TABLE 9

Isotope Effects in Metabolic Reactions of Serine and Formate. The Utilization of Deuterium Relative to Carbon is given by the Retention Factor F_D. Complete Retention of Deuterium would give $F_D = 1$. Calculated Average Values are Indicated by an Asterisk

Labelled compound used	*Metabolic product*	F_D	*References*
$^{14}CH_2OH \cdot CH(NH_2) \cdot CO_2H$ $CD_2OH \cdot CH(NH_2) \cdot CO_2H$	Methyl groups of choline	0·93*	Elwyn, Weissbach, and Sprinson (1951); Elwyn, Weissbach, Henry, and Sprinson (1955)
$^{14}CH_2OH \cdot CH(NH_2) \cdot CO_2H$ $CD_2OH \cdot CH(NH_2) \cdot CO_2H$	Methyl group of thymine	0·78*	Elwyn and Sprinson (1954b)
	$C_2 + C_8$ of RNA adenine	0·34*	
	$C_2 + C_8$ of DNA adenine	0·31*	
	C_8 of DNA + RNA guanine	0·21	
DCO_2Na $H^{14}CO_2Na$	Methyl group of thymine	0·89	
	$C_2 + C_8$ of RNA adenine	0·16	
	$C_2 + C_8$ of DNA adenine	0·34	
DCO_2Na $H^{14}CO_2Na$	Methyl group of choline	0·98*	Ressler, Rachele, and du Vigneaud (1952)

other precursors of methyl groups and related compounds (Table 9). In the conversion of the hydroxymethyl group of serine into choline methyl groups, formate is excluded as an intermediate by the retention of 93 per cent of the original deuterium. The small isotope effect in this reaction, as well as in the conversion of formate into methyl groups, may be due to the absence of reactions involving the labelled C—H bonds in these metabolic pathways. Only 78 per cent of the original deuterium was retained in the conversion of deuterium into the methyl group of thymine, indicating either an unusually large isotope effect or oxidation of some of the labelled precursor to the level of formate. On the other hand, the incorporation of deuterium into C_2 and C_8 of the purine molecule is much too low to be due to an isotope effect, and indicates the transitory formation of an intermediate in which the carbon-bound deuterium has become exchangeable.

Transmethylation. In view of the large isotope effects which may be observed in reactions involving isotopic hydrogen, it seems important to reconsider the present evidence indicating that the biosynthesis of choline involves the transfer of three intact methyl groups from methionine. In the early work (du Vigneaud *et al.*, 1941) methionine

containing approximately 85 atom per cent excess deuterium in the methyl group was fed to rats over prolonged periods, and the deuterium content of the choline methyl groups was determined at various times, the limiting value being about 78 atom per cent excess. Although these results were believed to show the intact transfer of almost three CH_3 groups per choline molecule, an alternative explanation now seems possible. It may be deduced from the method of synthesis that the labelled methionine contained mainly CD_3- and CD_2H-methyl groups. Oxidation of such a mixture of labelled methyl groups to formaldehyde would result in the enrichment of deuterium in the product, since C—H bonds are ruptured more easily. Moreover, CD_3-labelled methionine would be preferentially retained, so that the combined effect would be the formation of CD_3-labelled methionine and CD_2O containing a higher isotope concentration than the fed labelled methionine, the maximum possible concentration being about 100 atom per cent excess D. If two of the choline methyl groups were formed from formaldehyde by a reductive methylation and the third by transmethylation, the maximum possible deuterium concentration in a choline methyl group would be $7/9 \times 100 = 78$ atom per cent excess D, which is very similar to the observed value.

The intact transfer of methyl groups has also been deduced from the fact that L-methionine labelled with both ^{14}C and deuterium gives rise to choline and creatine methyl groups having the same ^{14}C:D isotope ratio as the precursor (KELLER, RACHELE and DU VIGNEAUD, 1949). Since CD_3-labelled methionine would, however, be oxidized more slowly than the $^{14}CH_3$-compound (RACHELE *et al.*, 1955), one might have expected a preferential utilization of the former for transmethylation and hence an *increased* D:^{14}C ratio in the product. The constant D:^{14}C ratio may thus be the result of compensating reactions. This possibility could be eliminated only by experiments with methionine labelled intramolecularly in the methyl group e.g. $^{13}CD_3$ (RACHELE *et al.*, 1955).

That the mechanism of biosynthesis of the three choline methyl groups may not be identical is suggested by recent work with folic-acid-deficient rats, which were unable to synthesize choline from aminoethanol and methionine, although transmethylation from methionine to dimethylaminoethanol was not impaired (STEKOL, WEISS and ANDERSON, 1955). At present, therefore, the possibility remains that one or two of the choline methyl groups are synthesized other than by transmethylation.

Note added in proof. The transfer of the intact methyl group of methionine to choline and creatine has now been demonstrated conclusively in elegant experiments with intramolecularly labelled $^{14}CD_3$ — methionine (DU VIGNEAUD, RACHELE and WHITE, 1956).

QUANTITATIVE CONSIDERATIONS

1. *Isotope-dilution technique*

The principle of this method which is of application to a wide variety of problems may be stated as follows. When a labelled compound (mass M and isotope concentration x) is diluted by mixing with a mass M' of its nonisotopic counterpart, the isotope concentration y of the resulting mixture will be $\left(\frac{M}{M+M'}\right)x$. Since x and y can be measured and M is known, it is possible to use this equation to calculate an unknown mass M' of the unlabelled sample originally present, i.e. $M' = M\left(\frac{x-y}{y}\right)$. Similarly if the mass of the labelled compound is unknown, then $M = \frac{M'y}{x-y}$ and this has been termed inverse isotope dilution. A discussion on the general theory and precision of these techniques is given by RITTENBERG and FOSTER (1940), RADIN (1947) and GEST, KAMEN and REINER (1947). The advantage of the method is that once the labelled compound and its non-isotopic compound have been thoroughly mixed, quantitative isolation is not necessary so that rigorous but wasteful purification procedures can be used to isolate a small representative sample for measurement of the isotope concentration y. The absolute isotopic purity of the latter sample is essential, although this is not always easy to accomplish (see KESTON, UDENFRIEND and CANNAN, 1949).

In its simplest form, the isotope-dilution technique may be illustrated in the determination of the equilibrium constant of the chymotrypsin-catalysed hydrolysis of benzoyl-L-tyrosylglycinamide (I).

$$\begin{array}{l} \qquad\qquad\quad CH_2\cdot C_6H_4OH \\ \qquad\qquad\quad | \\ C_6H_5\cdot CO\cdot NH\cdot CH\cdot CO_2H + {}^{15}NH_2\cdot CH_2\cdot CONH_2 \\ \qquad\qquad\quad \Uparrow\Downarrow \\ \qquad\qquad\quad CH_2\cdot C_6H_4OH \\ \qquad\qquad\quad | \\ C_6H_5\cdot CO\cdot NH\cdot CH\cdot CO{}^{15}NH\cdot CH_2\cdot CONH_2 + H_2O \\ \qquad\qquad\quad (I) \end{array}$$

Known amounts of benzoyl-L-tyrosine and [^{15}N] glycinamide were incubated with the enzyme solution, the reaction was stopped, a known amount of nonisotopic (I) was added, and a pure representative sample was isolated. The isotope concentration in this latter sample completes the data required to calculate the amount of (I) present at the end of the reaction. Thus, the molar amounts of reactants and products present under equilibrium conditions are known so that the

equilibrium constant may be calculated (FRUTON, JOHNSTON and FRIED, 1951). Apart from its use as an analytical procedure, such as the micro-estimation of histamine (SCHAYER, KOBAYASHI and SMILEY, 1955); amino acids (KESTON and LOSPALLUTO, 1951; BARKER, HUGHES and YOUNG, 1952) and pyrimidines (FRESCO and WARNER, 1955), the principle of isotope dilution has also been applied to the quantitative estimation of metabolic reaction rates which is discussed below.

2. *Determination of the rates of metabolic reactions*

2. 1. *Metabolic pools and turnover rates*

The concept of metabolic pools is implicit in the view of the living cell as a system in dynamic equilibrium which was elaborated by SCHOENHEIMER, RATNER and RITTENBERG (1939) as a result of their fundamental tracer experiments on protein metabolism. According to this view, which is now generally accepted, most tissue constituents are continuously degraded and resynthesized. The metabolic pool of an animal, organ, or tissue has been defined as that mixture of compounds derived from the diet and from the breakdown of tissues which is utilized for the continuous synthesis of tissue constituents (SPRINSON and RITTENBERG, 1949). The metabolic pool is further subdivided into pools of individual compounds such as glucose (FELLER, STRISOWER and CHAIKOFF, 1950), or groups of related substances, as for example compounds of the nitrogen pool used for protein synthesis (SPRINSON and RITTENBERG, 1949). The term "pool" has also been used to indicate the amount of a given substance synthesized or metabolized in unit time (ANKER, 1948). It seems desirable, however, to restrict the use of the term "pool" to the definition given previously.

The quantitative estimation of metabolic pools is usually carried out under "steady-state" conditions where the rate of biosynthesis of the metabolite being studied equals the rate of its breakdown, and the size of the metabolic pool remains constant. By determining the isotope concentration in the metabolite at various time intervals following the administration of a known tracer dose, it is possible to calculate the pool size and its turnover rate. Thus the extrapolation of the isotope concentration—time curve to zero time gives the dilution of administered isotope which would have been observed if the labelled compound had mixed instantaneously and completely with the metabolic pool. The size of the pool is then obtained by multiplying the amount of labelled compound given by this dilution factor, whilst its turnover is measured by the rate of decrease in the isotope concentration with time.

Usually it is not possible to correlate the metabolic pool of a substance with an identifiable compound in the body. Thus, the glucose pool in

the rat considerably exceeds the amount of free glucose (FELLER, STRISOWER and CHAIKOFF, 1950), probably because some of the glycogen is in a state of very rapid equilibrium with free glucose. Similarly, the uric acid pool of patients suffering from gout is larger than can be accounted for by free uric acid, and includes a portion of the solid-phase urates (BENEDICT, FORSHAM and STETTEN, 1949). The effective metabolic pool of a substance metabolized by a particular reaction may, however, be considerably less than the total amount present in the body, owing to permeability barriers. Thus, the exchange of plasma and liver glycine is fast while the exchange of plasma and muscle glycine is slow (HENRIQUES, HENRIQUES and NEUBERGER, 1955). It is not surprising, therefore, the the glycine pool immediately available for conjugation with small doses of benzoate corresponds approximately to the free glycine present in liver, kidney and intestine (ARNSTEIN and NEUBERGER, 1951), since hippuric-acid synthesis in most species take place in the liver and kidney.

There has been considerable discussion recently about the definition of "turnover," which at present is being used with two different meanings. As originally defined by ZILVERSMIT, ENTENMAN and FISHLER (1943), the turnover rate of a substance in a tissue is the amount of that substance which is replaced in the tissue in unit time, while the turnover time is the time required for the renewal of an amount of substance equal to that present in the tissue, i.e. equal to the metabolic pool. It has been pointed out by KLEIBER (1955) that turnover rate is frequently used to describe that fraction of the metabolic pool which is renewed in unit time, i.e. the reciprocal of turnover time, which is thus equal to the turnover rate as defined by ZILVERSMIT *et al.* (1943) *divided by the pool size*. MAWSON (1955) favours this new definition of turnover rate and suggests that the original one be now termed flux rate. It should be noted, however, that the rate of transfer of a labelled compound across a membrane may bear no direct relationship to the absolute flux through the membrane (EDWARDS and HARRIS, 1955); and the introduction of a new term, such as flux rate, can only add to the confusion. It seems preferable to adopt the terminology of ZILVERSMIT (1955) in which turnover rate remains as originally defined and the rate at which the metabolic pool is renewed (the Kleiber definition) is termed the fractional turnover rate.

2. 2. *The interpretation of isotope dilution under conditions of continuous feeding*

The endogenous synthesis of a compound may be estimated from the dilution of isotope concentration which occurs when a known amount of the labelled compound is fed as a component of the diet over a definite period of time. It is sometimes possible, and more convenient,

to isolate the compound itself or a suitable derivative from the urine rather than directly from the body tissues. This is a particularly useful procedure since the intracellular concentrations of many metabolic intermediates are extremely low. The endogenous rate of acetate formation has been calculated in this way from the isotope content of urinary acetyl derivatives which are excreted when labelled acetate is fed simultaneously with foreign amines. The production of acetic acid by the rat was found to be approximately 15–20 mmoles per day per 100 g body weight (BLOCH and RITTENBERG, 1945). Since these foreign amines are acetylated also by pyruvate (ANKER, 1948; 1950) the above isotope experiments really measure the combined acetate and pyruvate pools. The size of these pools is, however, smaller than would be expected from the catabolism of dietary carbohydrate and fat, which may therefore not be in complete equilibrium with dietary acetate. Indeed, from the results of long-term feeding experiments (see below) with labelled alanine (ARNSTEIN and STANKOVIĆ, 1956; ARNSTEIN and KEGLEVIĆ, 1956) it may be calculated that the pyruvic acid pool amounts to about 38 mmoles per day per 100 g body weight.

Instead of measuring the isotope concentration in derivatives of metabolites which are excreted in the urine, it is often possible to obtain representative samples of a compound from tissue constituents such as protein. In this case, however, it is necessary to ensure that the feeding period is long relative to the turnover time of the tissue component used to "trap" the metabolite. The rates of biosynthesis of serine and glycine have been investigated by this technique, which involved the feeding of diets containing known amounts of ^{14}C-labelled glycine and serine to rats over periods extending up to 39 days, and estimating the isotope concentration of glycine and serine isolated from the visceral proteins, which have a relatively fast turnover. The rate of glycine formation was 2·5 mmoles per day per 100 g body weight, whereas that of serine was 3·5 mmoles, indicating that glycine may be derived largely from serine (ARNSTEIN and NEUBERGER, 1953b). It is noteworthy that the dilution of isotope observed in urinary hippuric acid following the injection of ^{14}C-labelled glycine and benzoic acid into rats (SHEMIN, 1946) corresponds to only about 1 mmole per day per 100 g body weight. This lower estimate is probably due to the fast conjugation of glycine with benzoic acid, resulting in incomplete mixing of the injected labelled glycine with the body pool of glycine. Long-term feeding experiments have also shown that the rate of glycine formation was markedly reduced in folic-acid-deficient animals, but that other vitamin deficiencies (vitamin B_{12} or ascorbic acid) had no effect (ARNSTEIN and STANKOVIĆ, 1956).

One interesting observation which emerges from these long term feeding experiments is the constancy of the rate of glycine biosynthesis,

which is independent of the level of glycine in the diet (ARNSTEIN and NEUBERGER, 1953b). It thus appears that the constant levels of glycine and probably also other biosynthetic products are maintained mainly by regulation of the rate of disposal rather than by regulation of the rate of formation.

3. *Precursor-product relationships in the whole animal*

ZILVERSMIT, ENTENMAN and FISCHLER (1943) have presented the mathematical basis of the relationship, under steady-state conditions, between the specific activity of a precursor and its product over a short period of time subsequent to a single injection of the labelled compound into the animal. The specific activity—time curves of precursor and product may be used to calculate turnover times and rates. In addition, the relationship between these experimentally determined curves can be compared with that predicted by theory, and in this way it is possible to identify the precursor of a compound from a number of suspected ones. For example, after injection of $^{32}PO_4^{---}$ into rats, the only part of the acid-soluble phosphorus fraction which fulfilled the mathematical criteria for an immediate phosphorus precursor of lecithin and kephalin was the ethanol-precipitable phosphorus. This latter fraction was identified as being mainly α and β glycerophosphates (POPJÁK and MUIR, 1950). A similar conclusion was reached by ZILVERSMIT, ENTENMAN and CHAIKOFF (1948), using dogs as experimental animals. The specific activity—time curves for lactose and glyceride-glycerol in milk indicate that glucose (from which lactose is formed rapidly) is the carbohydrate precursor of glycerol in the mammary gland (POPJÁK, GLASCOCK and FOLLEY, 1952). This procedure, however, while yielding some quantitative information on precursor-product relationships, provides no information as to whether the labelled compound is the only precursor.

In certain cases the quantitative significance of a precursor may be estimated by feeding the labelled precursor to the animal over a relatively long period until the isotope concentration in the metabolic products is at a maximum with respect to the labelled compound fed. For example, if the synthesis of a product B, which is omitted from the diet, takes place exclusively from a precursor A, the specific activity of B will then be identical with that of A even although the conversion involves a series of intermediates, X, Y and Z.

$$\begin{array}{ccccccccccc} & & & & C & & & & & & \\ & & & \nearrow & & & & & & & \\ \text{endogenous synthesis} & \rightarrow & A & \rightarrow & X & \rightarrow & Y & \rightarrow & Z & \rightarrow & B \leftarrow P \\ & & & \searrow & & & & & & & \\ & & & & D & & & & & & \end{array}$$

Neither the formation of other products *C* and *D*, nor dilution of labelled *A* by endogenous synthesis, will affect the interpretation of the experiment if the specific activity of *B* is compared with that of *A* isolated from the same tissue or compound, e.g. two amino acids isolated from the same protein sample. On the other hand, biosynthesis of *B* from an alternative precursor *P* will result in dilution of the specific activity of *B* and cause a reduction in the ratio of specific activities *B*:*A*. The efficiency of glycine and serine as C-1 precursors in the *de novo* synthesis of methyl groups has been compared by this method (ARNSTEIN and NEUBERGER, 1953a). When a methionine- and choline-free diet containing L-[β-^{14}C]-serine was fed to rats over a period of 20–39 days, the radioactivity of the methyl carbon of methionine was about 70 per cent of that of the β carbon of serine isolated from the same tissue protein sample. In contrast, an equimolar amount of [α-^{14}C]glycine under similar conditions was found to be a much less efficient methyl group precursor, and it was concluded that serine is a major precursor of newly-synthesized methyl groups. In a similar manner, the quantitative importance of the methyl group of methionine for choline and creatine biosynthesis by transmethylation had been previously shown by the feeding of deuterium-labelled methionine over a period of 14 weeks (DU VIGNEAUD *et al.*, 1941).

ALTERNATIVE METABOLIC PATHWAYS

When a compound can be metabolized by more than one pathway the quantitative contribution of such alternative pathways has to be assessed. Glucose, for example, serves not only as a major source of energy, but also as a direct precursor of several C_6 compounds such as glucosamine (TOPPER and LIPTON, 1953; ROSEMAN, MOSES, LUDOWIEG and DORFMAN, 1953), glucuronic acid (DOUGLAS and KING, 1953; EISENBERG, 1955) and galactose (DIMANT, SMITH and LARDY, 1953). Moreover, different mechanisms of glucose catabolism have been demonstrated in various species of micro-organisms (GUNSALUS, HORECKER and WOOD, 1955; RACKER, 1954; WOOD, 1955) and in mammals two alternative mechanisms of glucose breakdown have been known for some time (see reviews by STUMPF, 1954; DICKENS, 1956; WOOD, 1955).

The detailed reactions of a metabolic pathway are often more readily demonstrated *in vitro* than *in vivo*, but their quantitative importance can be determined only by *in vivo* experiments, since the regulated metabolic activity of the intact organism may determine the amount of a substance metabolized by alternative routes. Occasionally, a pathway indicated by *in vitro* experiments may be absent or quantitatively unimportant in the whole organism. This may explain the

differences in glycine metabolism under *in vitro* and *in vivo* conditions (ARNSTEIN, 1954).

The isotopic tracer technique probably offers the best experimental approach to the problem of determining the importance of alternative pathways *in vivo*, but the proper design and interpretation of such experiments may be very difficult, as has been pointed out by WOOD (1955) in a thorough discussion of glucose metabolism.

IX. 1. *Glucose metabolism*

The detailed catabolism of the biosynthetic products of glucose, such as glucosamine and galactose, is at present not known, and the possible contribution of these substances to the overall metabolism is not clear, but is unlikely to be of quantitative importance. Moreover, in some instances these substances may be reconverted into glucose rather than metabolized by a separate pathway. Thus, the reversibility of the formation of galactose 1-phosphate from glucose 1-phosphate (HANSEN and CRAINE, 1954) and the similar rates of oxidation of glucose and galactose *in vivo* (CARLETON, MISLER and ROBERTS, 1955) suggest rapid interconversion of the two sugars and a common metabolic pathway.

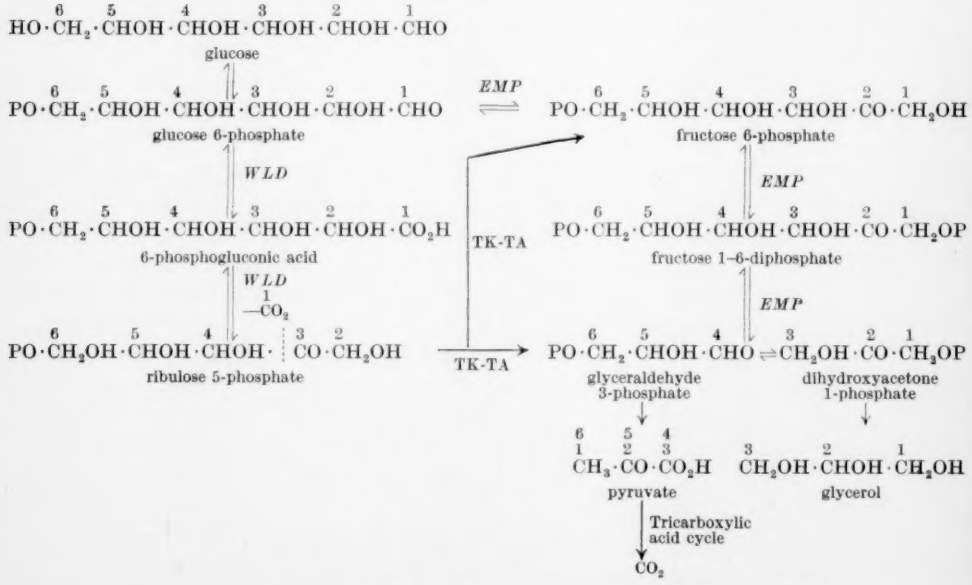


Fig. 5. The metabolism of glucose by alternative metabolic pathways. The glycolytic pathway is indicated by the abbreviation *EMP*, the oxidative pathway *via* 6-phosphogluconic acid by *WLD*. Reactions catalysed by transketolase (TK) and transaldolase (TA) convert pentose into triose + hexose (see Fig. 6). The numbers above individual carbon atoms refer to the numbering of glucose from which they are derived.

In certain micro-organisms special pathways of glucose metabolism exist (RACKER, 1954; GUNSALUS *et al.*, 1955), but in general there are two metabolic pathways by which glucose is broken down, ultimately to pyruvate and thence to carbon dioxide by the reactions of the tricarboxylic acids cycle (Fig. 5). In the glycolytic or Embden-Meyerhof-Parnas (EMP) pathway, glucose is converted into fructose 1:6-diphosphate which is cleft by aldolase to a mixture of dihydroxyacetone and glyceraldehyde phosphates. In the hexose monophosphate or Warburg-Lipmann-Dickens (WLD) pathway, glucose 6-phosphate is oxidized to 6-phosphogluconic acid which is converted into pentose phosphates. The catabolism of pentose by the reactions catalysed by transketolase and transaldolase (Fig. 6) results in the formation of

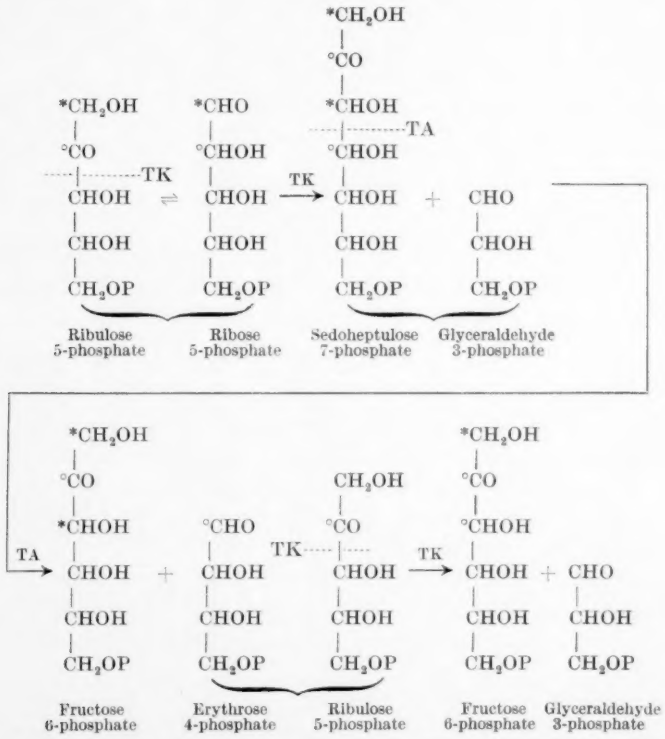


Fig. 6. Metabolism of pentose by transketolase (TK) and transaldolase (TA) reactions. Reactants are indicated by horizontal brackets and cleavage of carbon-carbon bonds by dotted lines. Pentose (ribose + ribulose) phosphate arises from hexose by the *WLD* pathway (Fig. 5).

1 molecule of triose and the resynthesis of 2 molecules of hexose monophosphate from 3 molecules of pentose. Triose phosphate, arising either by the EMP or the WLD pathway, is then oxidized to pyruvate,

which is further catabolized into carbon dioxide by the tricarboxylic acid cycle (see Fig. 3, p. 192).

There are three salient features which characterize the above alternative pathways of glucose catabolism with respect to the metabolic fate of the individual carbon atoms of the glucose molecule: (1) $C_{(1)}$ of glucose is converted directly into carbon dioxide by the WLD mechanism. The EMP pathway on the other hand converts this carbon atom into the methyl carbon of pyruvate, and carbon dioxide is produced only subsequently by the reactions of the tricarboxylic acid cycle. (2) Resynthesis of glucose by reversal of the EMP pathway does not affect the distribution of ^{14}C in labelled glucose, but resynthesis of hexose from pentose by the transketolase-transaldolase reactions results in the following redistribution of radioactivity originally present in $C_{(2)}$ and $C_{(3)}$ of glucose:

$C_{(2)}$ of hexose (indicated by C* in Fig. 6) $\rightarrow$
$C_{(1)}$ of pentose $\rightarrow$ $C_{(1)} + C_{(3)}$ of sedoheptulose $\rightarrow$

$C_{(1)} + C_{(3)}$ of hexose (activity of $C_{(1)} > C_{(3)}$ since two out of three molecules of labelled pentose are converted into hexose labelled in $C_{(1)}$, but only 1 molecule into hexose labelled in $C_{(3)}$);

$C_{(3)}$ of hexose $C_{(2)}$ of pentose (indicated by C° in Fig. 6) $\rightarrow$ C_2 of pentose $\rightarrow$ $C_{(2)} + C_{(4)}$ of sedoheptulose $\rightarrow$ $C_{(2)} + C_{(3)}$ of hexose (activity in $C_{(2)} > C_{(3)}$).

It may be noted that the formation of triose by this pathway is coupled with the resynthesis of hexose from pentose. Catabolism of [2-^{14}C] or [3-^{14}C] glucose by this mechanism should therefore lead to extensive redistribution of isotopic tracer in the unmetabolized glucose (e.g. that used for glycogen synthesis) in agreement with the above predictions. (3) Carbon atoms 1–3 of glucose are converted into triose and pyruvate only by the EMP pathway. In the WLD pathway, $C_{(2)}$ and $C_{(3)}$ of glucose are utilized, in the first place, for pentose synthesis. Pentose is then converted into hexose as discussed above, and $C_{(2)}$ and $C_{(3)}$ are thus oxidized to carbon dioxide by repetition of the WLD pathway.

These three major points of difference have been used to estimate the contribution of the EMP and WLD pathways in the intact organism and in isolated tissues, as described below.

1. 1. *The distribution of isotopic carbon in glycogen as an indicator of alternative metabolic pathways*

Since glycogen is synthesized directly from glucose 1-phosphate it may be used for "trapping" glucose from the metabolic pool (Wood, 1951). The distribution of isotope in glycogen corresponds closely to that in

the precursor glucose, as shown by the conversion of [1-^{14}C] glucose into glycogen containing 80–90 per cent of the total ^{14}C in carbon atom 1 and not more than 5 per cent in any of the other positions (Cook and Lorber, 1952; Hers, 1955).

Liver glycogen isolated from animals which had been injected with $NaH^{14}CO_3$ was labelled almost entirely in $C_{(3)}$ and $C_{(4)}$ of the glucose unit (Bernstein *et al.*, 1955). Only a small proportion of the labelled hexose monophosphate used for glycogen synthesis could therefore have originated by the WLD reactions, which would have resulted in redistribution of radioactivity from $C_{(3)}$ into $C_{(2)}$. In this type of experiment, however, glycogen deposition is enhanced by using fasted animals, which are fed glucose at the time of injection of $NaH^{14}CO_3$. Under these conditions, catabolism and resynthesis of glucose by the WLD pathway may well be slow compared with gluconeogenesis by reversal of the EMP reactions and glycogen formation. The distribution of isotopic carbon in glycogen may thus reflect the metabolic pathway used for *de novo* glucose synthesis rather than that for glucose catabolism. It is likely that the distribution of ^{14}C in glycogen synthesized by nonfasted animals from [2-^{14}C] glucose would be more informative about these alternative pathways. In this case, direct utilization of glucose would label glycogen only in $C_{(2)}$, catabolism to trioses and resynthesis by the EMP pathway would give equal labelling in $C_{(2)}$ and $C_{(5)}$, whilst catabolism and resynthesis by the WLD pathway would give equal labelling in $C_{(1)}$ and $C_{(3)}$ of the glucose residues of glycogen.

1. 2. *Calculations based on the conversion of labelled glucose into carbon dioxide*

The relative contribution of the alternative pathways to the oxidation of glucose by tissue slices has been calculated from a comparison of the conversion of [1-^{14}C] and uniformly ^{14}C-labelled glucose ([U-^{14}C] glucose) (Bloom, Stetten and Stetten, 1953), on the assumption that carbon dioxide is formed *only* from $C_{(1)}$ of glucose by the WLD pathway.

On this assumption, kidney slices were calculated to metabolize 91 per cent of the glucose by the EMP pathway, whilst liver slices metabolized only 23 per cent by this route. In similar experiments with [1-^{14}C] and [6-^{14}C] glucose, it was estimated that 36 per cent of the glucose converted into carbon dioxide by liver slices was metabolized by the EMP pathway (Bloom and Stetten, 1953). The discrepancy between this result and the value obtained from the comparison of [1-^{14}C] and [U-^{14}C] glucose may indicate that some of the fundamental assumptions made in this type of experiment are not valid.

Thus, re-synthesis of hexose from pentose (Horecker, Gibbs, Klenow and Smyrniotis, 1954; Katz, Abraham, Hill and Chaikoff, 1955) would result in the conversion of $C_{(2)}$ and $C_{(3)}$ of glucose into

carbon dioxide, whilst $C_{(4)}$–$C_{(6)}$ of glucose may also contribute to the carbon dioxide pool, since triose originating from these carbon atoms by the WLD mechanism is presumably further metabolized to pyruvate and thence to carbon dioxide. Moreover, it has been pointed out by WOOD (1955) that only glucose molecules which are eventually converted into carbon dioxide are estimated, and in liver this is probably only a minor pathway.

Using the same experimental results as BLOOM *et al.* (1953) but a different method of calculation (KATZ, ABRAHAM, HILL and CHAIKOFF, 1954; KATZ, *et al.*, 1955) it has been concluded that in liver slices as much as 61–94 per cent of the glucose is metabolized by the EMP pathway. In this calculation, the yield of labelled carbon dioxide from all six carbon atoms of glucose by the EMP pathway was compared with that of $C_{(1)}$ by the WLD pathway, and the validity of such a comparison has been questioned (WOOD, 1955). Nevertheless, the conclusion that only relatively small amounts of glucose are catabolized by the WLD mechanism is supported by the absence of any significant difference in the relative utilization of [1-^{14}C] and [U-^{14}C] glucose for the biosynthesis of several compounds related to pyruvate and the tricarboxylic acid cycle, including alanine, aspartic acid, and glutamic acid (KATZ *et al.*, 1955). On the other hand, liver slices incorporated more ^{14}C into fatty acids from [6-^{14}C] glucose than from [1-^{14}C] glucose (BLOOM and STETTEN, 1955), which would be indicative of a preferential conversion of glucose to acetate by the WLD pathway.

1. 3. *Calculations based on the conversion of different carbon atoms of glucose into* C_3 *compounds related to triose*

A different method of calculating the relative contribution of the alternative pathways of glucose metabolism, based on a comparison of the specific radioactivity of triose synthesized from glucose labelled in different positions, has been developed by BLUMENTHAL, LEWIS and WEINHOUSE (1954). Since glucose labelled in $C_{(1)}$, $C_{(2)}$ or $C_{(3)}$ gives rise to labelled triose only by the EMP pathway, the conversion of [1-^{14}C] glucose into lactate, pyruvate, or acetate was used to calculate the amount of glucose converted into these products by the EMP pathway, allowance being made for endogenous dilution by using [U-^{14}C] glucose in control experiments. The validity of this method depends on the equal utilization of the two triose phosphates (dihydroxyacetone phosphate and glyceraldehyde phosphate) produced by glycolysis for pyruvate synthesis, and on the complete mixing of trioses from the EMP and WLD pathways (WOOD, 1955). The unequal labelling of $C_{(3)}$ and $C_{(4)}$ of glucose synthesized from [1-^{14}C] glycerol (SCHAMBYE, WOOD and POPJÁK, 1954) suggests, however, that the first assumption may not be entirely valid, since reversal of this pathway would result

in the preferential utilization of $C_{(1)}$ of glucose for glycerol biosynthesis. Pyruvate would then be derived preferentially from $C_{(4)}$–$C_{(6)}$ of glucose even by the EMP mechanism, and the contribution of the WLD pathway may thus be overestimated.

This method has been used to determine the pathways of glucose metabolism in *Pseudomonas fluorescens* (LEWIS, BLUMENTHAL, WEINRACH and WEINHOUSE, 1955), and in yeasts (BLUMENTHAL *et al.*, 1954). Aerobically, 0–30 per cent and 30–50 per cent of the glucose was estimated to be metabolized by the WLD pathway by *Saccharomyces cerevisiae* and *Torulipsis utilis*, respectively, whilst anaerobically at least 95 per cent was catabolized by the EMP pathway. The biosynthesis of alanine, which is closely related to metabolic pyruvate, from dietary [1-^{14}C] and [U-^{14}C] glucose, has been used in similar experiments to estimate the pathways of glucose catabolism in young rats (ARNSTEIN and KEGLEVIĆ, 1956). The prolonged experimental period during which the labelled glucose was fed in this case would probably minimize some of the factors, such as differences in reaction rates and metabolic pools of intermediates, which may complicate the interpretation of short-term experiments (WOOD, 1955). Alanine biosynthesis from [1-^{14}C] glucose was identical with that from [U-^{14}C] glucose and, in agreement with the results of KATZ *et al.* (1955), and BLOOM *et al.* (1953), it was concluded that the WLD pathway is quantitatively unimportant for the conversion of glucose into pyruvate by the intact animal.

2. *Biosynthesis of glycine*

The metabolic importance of glycine as a precursor of a wide variety of nitrogenous compounds is now well established, but relatively little is known about the mechanism of its biosynthesis from nonnitrogenous precursors (for a recent review, see ARNSTEIN, 1954).

In animals glycine is a nutritionally dispensable amino acid (ROSE, BURR and SALLACH, 1952) and the extensive metabolic interconversion of serine and glycine (SHEMIN, 1946; SAKAMI, 1948) suggests the possible existence of two alternative pathways of glycine biosynthesis. One pathway (reactions 1 and 2, Fig. 7) would involve the initial formation of serine from a C_3 percursor, followed by loss of the hydroxymethyl

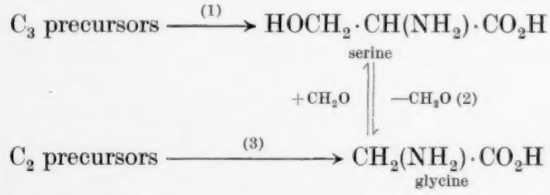


Fig. 7. Possible alternative pathways of glycine biosynthesis.

group of serine; the other (reaction 3) would consist of the direct synthesis of glycine from a C_2 compound. Both reactions may, of course, occur simultaneously, and it is noteworthy that glyoxylic acid is converted into glycine (WEINHOUSE and FRIEDMANN, 1951), whilst pyruvate (ANKER, 1948) and glucose (ARNSTEIN and KEGLEVIĆ, 1956) can serve as precursors of serine.

The problem of glycine biosynthesis is further complicated by the existence of two metabolic cycles (Fig. 8, *A* and *B*), both of which result in the indirect interconversion of glycine and serine. In cycle *A*,

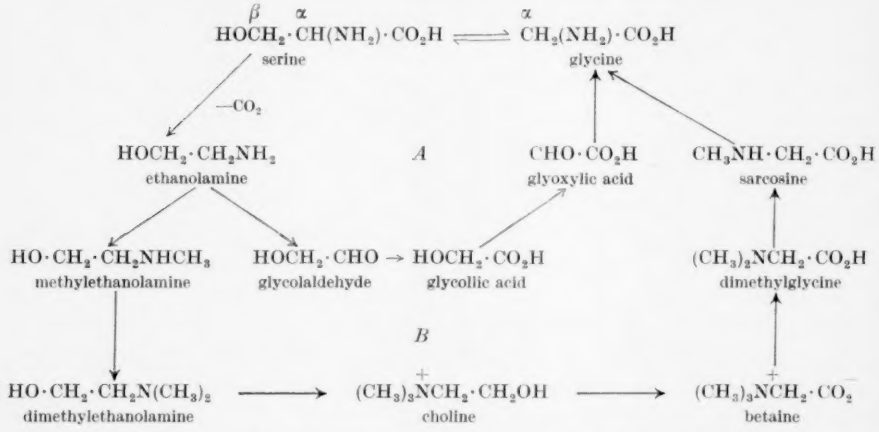


Fig. 8. Metabolic pathways leading to the interconversion of serine and glycine.

serine is first decarboxylated to ethanolamine, which is deaminated, oxidized to glyoxylic acid, and thus converted into glycine (WEISSBACH and SPRINSON, 1953). In cycle *B*, ethanolamine is methylated to choline, which after oxidation to betaine is successively demethylated to dimethylglycine, sarcosine and finally glycine (SOLOWAY and STETTEN, 1953).

The formation of glycine from serine by cycle *A* results in the loss of the nitrogen atom and the conversion of the α and β carbon atoms of serine into the carboxyl and α carbon atoms of glycine, respectively. In cycle *B*, on the other hand, the nitrogen atom of serine is retained and the β carbon atom becomes the carboxyl carbon atom of glycine. On the basis of these differences it is possible to assess the approximate quantitative contribution of the two cycles to the conversion of serine into glycine.

Since [β-^{14}C] serine gives rise to glycine containing considerably more radioactivity in the α carbon atom than in the carboxyl carbon atom (ELWYN and SPRINSON, 1954a), the contribution of cycle *B* is evidently of little importance. It is likely that the rate-limiting

reaction in this pathway is the methylation of dimethylethanolamine to choline, since both choline and betaine are readily converted into glycine, whereas dimethylethanolamine is only a poor precursor (SOLOWAY and STETTEN, 1953).

The reactions of cycle *A* would lead to the incorporation of ^{14}C from [α-^{14}C] glycine into the carboxyl groups of both serine and glycine. Even after prolonged feeding of [α-^{14}C] glycine to young rats, only about 10 per cent or less of the total ^{14}C was found in the carboxyl group of glycine (H. R. V. ARNSTEIN, unpublished results) or serine (ARNSTEIN and NEUBERGER, 1953b; see also p. 184). It is concluded therefore, that this pathway is also only of minor significance compared with the direct glycine-serine interconversion (Fig. 7, reaction 2).

In quantitative experiments it has been found that the rate of serine biosynthesis by the rat exceeds that of glycine by about 50 per cent (ARNSTEIN and NEUBERGER, 1953b; see also p. 207). The assumptions made in these experiments have been discussed in detail elsewhere (ARNSTEIN, 1954), and while the absolute figures may require modification it is clear that the rate of glycine biosynthesis is insufficient to account for the biosynthesis of serine entirely from glycine. On the other hand, the direct formation of glycine entirely from serine would be compatible with the quantitative estimates of the rates of biosynthesis of these amino acids.

Since the metabolic interconversion of glycine and serine requires pteroylglutamic acid (ELWYN and SPRINSON, 1950), the rate of glycine biosynthesis by normal and pteroylglutamic-acid-deficient rats was compared in order to determine the importance of the glycine-serine reaction for the biosynthesis of glycine. A dietary deficiency of pteroylglutamic acid reduced the rate of glycine biosynthesis by the rat to about 60 per cent of the normal value (ARNSTEIN and STANKOVIĆ, 1956). The conversion of ^{14}C-labelled glucose into glycine was reduced to a similar extent, but serine biosynthesis from glucose was not affected (ARNSTEIN and KEGLEVIĆ, 1956), indicating that glycine is not an intermediate in the biosynthesis of serine. The conversion of glucose into serine *via* a C_3 precursor (reaction 1, Fig. 7) can thus account quantitatively for serine biosynthesis, whilst the serine-glycine reaction (reaction 2) apparently synthesizes at least 40 per cent of the glycine normally derived from nonnitrogenous precursors. This figure probably represents only a minimum value, since the serine-glycine reaction was only reduced and not completely inhibited in these experiments.

Whether C_2 precursors also contribute to glycine biosynthesis remains uncertain, although the formation of glycine from glyoxylic acid has been demonstrated *in vivo* (WEINHOUSE and FRIEDMANN, 1951; WEISSBACH and SPRINSON, 1953). Since this reaction is reversible, as shown by the deamination of glycine to glyoxylic acid in liver

homogenates (NAKADA and WEINHOUSE, 1953), its occurrence *in vivo* should result in a change in the ^{14}C:^{15}N ratio of doubly-labelled glycine. It has been found, however, that in the intact rat, this ratio remains unchanged for many hours (ARNSTEIN and NEUBERGER, 1951). It is concluded, therefore, that metabolism of glyoxylic acid to glycine *in vivo* is either irreversible or quantitatively insignificant (possibly because only small amounts of glyxoylic acid may be normally available for glycine biosynthesis), and that the major part of glycine is normally synthesized from serine.

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THE X-RAY CRYSTAL ANALYSIS OF BONE

S. M. Clark and J. Iball

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5

THE X-RAY CRYSTAL ANALYSIS OF BONE

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I. Introduction

I. 1. *General*

During the past thirty years since the first x-ray crystal analysis studies of bone were made, some hundreds of papers have been written on this subject, and the results have, on the whole, been rather disappointing. No very clear picture of the structure of bone is possible even now. In fact it is still not possible to state, without fear of contradiction, what is the exact substance, or substances, which makes up the inorganic part of normal bone. In the early investigations of the crystal structure of bone there was a suggestion that the results obtained might lead to a better understanding of the differences between normal healthy bone and diseased bone. In view of the uncertainty concerning so-called normal bone, it is not surprising to find that, when a comparison is made between normal bone and diseased bone, definite conclusions about the differences in the ultimate structure cannot be reached in many cases. A study of the literature on the structure of normal and diseased bone reveals many conflicting statements and some apparently irreconcilable results.

Several review articles on bone have been published, e.g., Huggins (1937), Fankuchen (1945), Dallemagne (1950), and Carlström and Engström (1956). Most of these, however, have a physiological or chemical bias and the results from x-ray diffraction form only part of the report. It is the purpose of the present report to review the results obtained by means of x-ray diffraction by previous investigators in this field and to present some new results. In conclusion an attempt will be made to assess the present position and to suggest some lines of investigation for the future.

Before the information obtainable by x-ray diffraction methods is discussed, it may be useful to describe, in the first place, what is already known about bone as a tissue and how bones such as the femur develop with age; and in the second place, to outline the various methods of x-ray diffraction which are applicable to the study of bone.

Bone consists of a basic organic substance which is impregnated with inorganic salts, the ratio by weight of these being approximately

* British Empire Cancer Campaign Research Fellow.

35 per cent organic to 65 per cent inorganic. X-ray diffraction techniques can not only give information about total, intact bone, but can also be applied separately to the inorganic and organic portions of bone. Much more work has been carried out on the inorganic portion of bone than on the organic part. The latter consists mainly of collagen, which is now being intensively studied by several different techniques. Bone is not the most suitable material for investigating collagen, and the results from studies of other collagenous material are likely to furnish a better understanding of the structure of the organic constituent of bone than is a direct attack on the bone material itself.

Bone tissue contains cells, called osteocytes, and intercellular substance. The intercellular substance is composed of fibrils and a cementing substance which binds them together and which contains most of the mineral salts. Mature bone is laid down in thin layers or lamellae. There are two types of bone, distinguishable microscopically, which differ in the arrangement of the lamellae contained in them. The first is called cancellous bone or "spongy" bone, the latter name describing its appearance well. The second is called compact bone. Most of the lamellae in compact bone are arranged in groups of cylindrical systems, the centre of each containing a canal with blood vessels. These cylindrical formations, called Haversian systems, are closely packed together, and the spaces between are occupied by irregularly arranged lamellae, called interstitial lamellae.

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I. 2. *The development of bone*

The ultimate structure of bone is more understandable if one knows how it is formed, so a brief account is given of the process of growth in so far as it is relevant to the crystal structure.

Much of the x-ray crystal analysis work has been carried out on long bones, e.g. the femur of various animals, and in the case of these bones a complete cartilage model of the bone is first produced in the developing animal. This cartilage is not changed into bone but is gradually replaced by bone.

The process by which the cartilage becomes replaced by bone is thought to take place in the following way. The cartilage model of the bone develops and increases in length and in width, the first by the mechanism of interstitial growth, and the second by the appositional mechanism, i.e. the formation of new layers of cartilage on the surface of the model. The interstitial growth tends to occur nearer the end of the model than the midshaft, and thus the cartilage in the midshaft has time to mature. After the process of maturation the intercellular substance in this area becomes calcified and, as always happens with calcified cartilage, the cells gradually die. The intercellular substance

therefore begins to break up, and cavities are left near the middle of the model.

While this is going on, conditions on the outer surface of the midshaft of the model are becoming favourable for the production of bone, and accordingly a thin layer of bone is laid down round the model. This layer of bone is covered by the periosteum, which is in fact responsible for the laying down of the bone. As the calcified cartilage in the midshaft of the model disintegrates, the process of bone formation begins to spread from the periosteum towards the centre of the model. When the osteogenic cells reach the interior of the midshaft they are said to constitute a centre of ossification, in this case the diaphyseal centre of ossification. This stage is reached in the human femur after about eight weeks of intrauterine life. The bone intercellular substance is laid down on what remains of the calcified cartilage and, since this is in the form of an irregular network with relatively large spaces, the bone formed here is cancellous or "spongy" bone. Gradually the cartilage cells next to this bone begin to mature and the same process, resulting in the formation of more bone, is repeated. Thus the bone slowly extends along the shaft of the cartilage model.

While this bone is being formed from the middle towards the ends of the model, the periosteum continues to add further bone to the sides of the model. When this bone becomes thick enough the cancellous bone inside it is superfluous and begins to dissolve away, leaving the bone with a central cavity called the marrow cavity.

In this manner the whole of the shaft of the model becomes ossified, but the two ends are still cartilage. Eventually two additional centres of ossification (epiphyseal centres of ossification) appear in the ends of the model, and the formation of bone occurs as before and spreads outwards from these two centres. Finally only a thin layer of cartilage is left at each end of the bone, between the bone resulting from the diaphyseal centre of ossification and the bone from the epiphyseal centre. This is called the epiphyseal disc and it persists until the longitudinal growth of the bone is completed. The continued interstitial growth in the epiphyseal disc is in fact responsible for further longitudinal growth of the bone. The epiphyseal disc tends to increase in thickness due to interstitial growth, but at the same time the cycle of calcification of the cartilage, its death and replacement by bone, takes place on the diaphyseal side of the disc, which tends to reduce its thickness. In this way the length of the bone increases.

In addition to increasing in length, the femur increases in width during the growing period. This latter process is achieved by new layers of bone being laid down on the outer surface of the shaft, while at the same time some bone is dissolved away from the inside

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of the shaft. Thus, although the shaft as a whole becomes wider, its walls do not become too thick, and in fact the marrow cavity also widens.

Usually the bone in the shaft of long bones is in the form of Haversian systems with interstitial lamellae. As the process of widening the bone slows up, the bone being laid down under the periosteum tends to form layers round the whole shaft, the so-called circumferential lamellae. Similar layers tend to form on the inner surface of the bone.

Even after growth of the bone has ceased, there is continual replacement of the old bone by new bone—some of the Haversian systems die and others form, although probably not in the same position, to replace them. Parts of the older Haversian systems remain and they then form the interstitial lamellae between the new Haversian systems.

I. 3. *The inorganic constituents of bone*

The chemical and physical-chemical analyses of the mineral part of bone have not succeeded in identifying with any certainty the exact substance or substances present. That the main constituent is an apatite-like material is easily established; the difficulty arises because there are several closely related substances, viz. carbonate-apatite (the mineral dahllite), α-tricalcium phosphate, and hydroxyapatite, which would have the necessary properties for the bulk of the inorganic bone substance.

The apatites are a class of minerals having a hexagonal crystal lattice, and certain atoms or groups of atoms can be substituted without altering the lattice to an appreciable extent. The one which has been studied in greatest detail is fluorapatite. Its structure was determined first by Naray-Szabo (1930) and independently by Mehmel (1930, 1931). Their results were checked, and the atomic positions defined more exactly, by Beevers and McIntyre (1946). The size of the unit cell of the hexagonal crystal lattice is defined by the parameters $a = 9{\cdot}37 \pm 0{\cdot}01$ Å, $c = 6{\cdot}88 \pm 0{\cdot}01$ Å. The crystal units are composed of 10 Ca, 6 P, 2 F, and 24 O atoms $(Ca_3F)_2(PO_4)_6Ca_4$. A long list of possible substitutions in the apatite crystal lattice is given by McConnell (1938). The fluorine atoms may be replaced by chlorine to give chlorapatite, or by hydroxyl ions to give hydroxyapatite. If carbonate groups are incorporated in the lattice of hydroxyapatite then a carbonate-apatite is produced, one such mineral (dahllite) being represented by the formula $Ca_6(OH)_2(P, C)_6O_{24}(Ca, C)_4$. As is mentioned later, some investigators suggest that the carbonate which is present in bone is incorporated in an apatite lattice, while others suggest that it is present as carbonate. Part of the difficulty in achieving finality in this field is that most methods used to identify the mineral substances in bone require that the bone be treated in a way

which might have the effect of causing changes in those substances. X-ray crystal-analysis methods are usually free from this criticism, but in the case of bone there are special difficulties attaching to the x-ray method.

I. 4. *The organic constituents of bone*

The fibrous organic material is collagen, which belongs to the group of fibrous proteins containing white connective tissue, tendons, cartilage, elastoidin, etc. The fibres of collagen are visible in the optical microscope with diameters ranging from 20 to 200 μ. These fibres can be seen to be divided into what are known as primitive fibres, which have diameters in the range 2 to 10 μ. Primitive fibres have thinner components, fibrils, which may be too thin (a few hundred Ångstroms) to be resolved in the optical microscope, and it is necessary to use an electron microscope. Fibrils, however, are composed of still thinner "filaments," and it is the arrangement of the molecular polypeptide chains in these filaments which is revealed by x-ray diffraction studies on collagen.

In addition to the fibrous material there is an organic matrix or ground substance. This matrix, which is more or less amorphous, contains mucopolysaccharides, e.g. hyaluronate, chondroitin sulphate, and acts as the cement between the collagen fibres. The crystallites of inorganic material are embedded in this substance.

II. X-Ray-Diffraction Methods

The particular method used depends largely on the type of material, but to a certain extent it depends on the complexity of the crystal units. There are three main methods of x-ray diffraction, each having a distinct experimental technique.

II. 1. *Powder method*

In this method the material investigated is usually in the form of microscopic crystals which are arranged in a random manner. (In bone, however, the crystals are too small to be seen with the optical microscope.) For example, two such materials are a metal wire which is normally composed of small randomly oriented crystals, and an inorganic crystal ground to a powder and then formed into a thin rod with some amorphous gum such as gum tragacanth. Each set of planes in the crystal lattice of each small crystal grain will diffract a narrow beam of x-rays in a given direction, and the angle which the diffracted beam makes with the incident beam depends on the spacing of the planes. This is stated in the Bragg diffraction law

$$n\lambda = 2d \sin \theta$$

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when λ is the wavelength of the x-rays, d is the spacing of the planes, and θ is the angle which the primary beam makes with the lattice plane (2θ is the angle between the primary beam and the diffracted beam).

A diffracted beam will be detected only when the angle θ is such that the above relation is obeyed. In a mass of small crystals randomly oriented there will be crystals in the correct orientation for *every* lattice plane and therefore, with an experimental arrangement such as that indicated in Fig. 1, each set of lattice planes with a spacing d will have

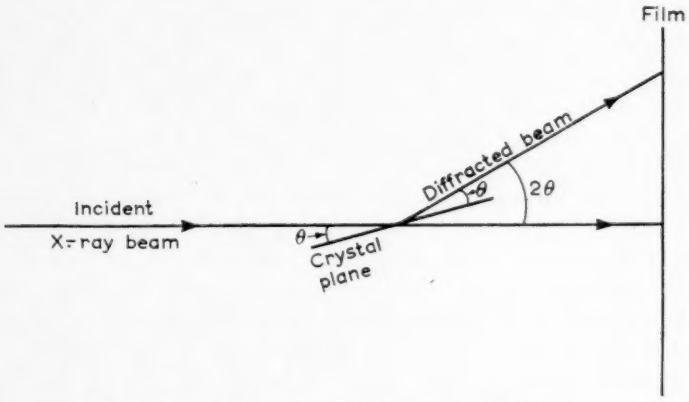

Fig. 1. Principle of x-ray diffraction with flat film.

all possible positions around the primary beam and still make an angle θ with it. Thus the diffracted beams for these particular planes will form a cone which has the direction of the primary beam as axis and an angle 2θ as its semiangle. Since each set of planes will give rise to its own cone of diffracted rays, each with a different semiangle, the complete pattern of diffracted beams will be a series of cones all having the primary beam direction as axis and each with a discrete value of semiangle between zero and 180°.

If a flat photographic film is arranged as in Fig. 1, so that its plane is perpendicular to the primary beam, the recorded pattern will be a series of circles with the point where the primary beam meets the film as centre. If D is the distance of the film from the specimen and R is the radius of a given ring on the film, then $\tan 2\theta = R/D$, and so from Bragg's law we can get a value of d, the spacing of the lattice planes which gave rise to the particular diffracted beam. A flat film will only record the few beams which have a value of 2θ below a certain value but, by surrounding the specimen with a cylindrical film, all the diffracted beams can be recorded. Since in this case only a strip of film is used, the record shows only parts of the circles shown on the flat

film. Since the size of the apparatus must be kept reasonable it will be seen that, if the crystal lattice is such that a very large number of planes can give diffracted beams, then the photographic record will be too crowded with lines for individual ones to be distinguished. Consequently it is not possible in general to use this method for crystal lattices other than the cubic and hexagonal ones. The information which can be obtained from powder diffraction diagrams varies a great deal, but the simplest and most important is the spacing of the lattice planes.

II. 2. *Single-crystal method*

When the material to be investigated exists as single crystals, then the individual beams diffracted from each set of planes of the lattice can be studied and complicated lattices can be investigated as well as simple ones. In order that each set of planes can be oriented to the primary beam so that the Bragg equation is satisfied, it is necessary to turn the crystal about an axis. As each set of planes of the lattice comes into the diffracting position the diffracted beam will make the appropriate angle 2θ with the primary beam but, in order to record them all with one crystal setting, it would be necessary to have a spherical film surrounding the crystal. In practice it is usual to have a cylindrical film approximately 3 cm diameter and 12 cm long; the crystal is then mounted about two or more axes in turn so that all the diffracted beams will be recorded. In this method it is possible to identify each diffracted beam and to measure the spacing of the corresponding planes but, more important, it is possible to measure the intensities of the beams. From the intensities it is possible to deduce, in many cases, the positions in the crystal lattice of the individual atoms of the substance. Several refinements of the single-crystal method have been devised in which the films are moved relative to the specimen while the exposure is being made. These are to simplify the interpretation of the pattern of spots which appear on the films. With a moderately sized lattice-unit cell in the monoclinic crystal system there may be as many as 3000 separate and distinct diffracted beams when copper K_α radiation is used.

II. 3. *Fibre diagrams*

In this case we have material which is usually much less crystalline than either a powdered crystal or a single crystal, but which gives an X-ray diffraction pattern which is in many respects half way between the powder diagram and the single-crystal one. If, for example, a metal wire is used as a specimen, it normally gives a powder diagram (of uniform circles if recorded on a flat plate); but if it is stretched many of the circles cease to be uniform and consist of "arcs" of high density separated by regions of low density. This shows that the

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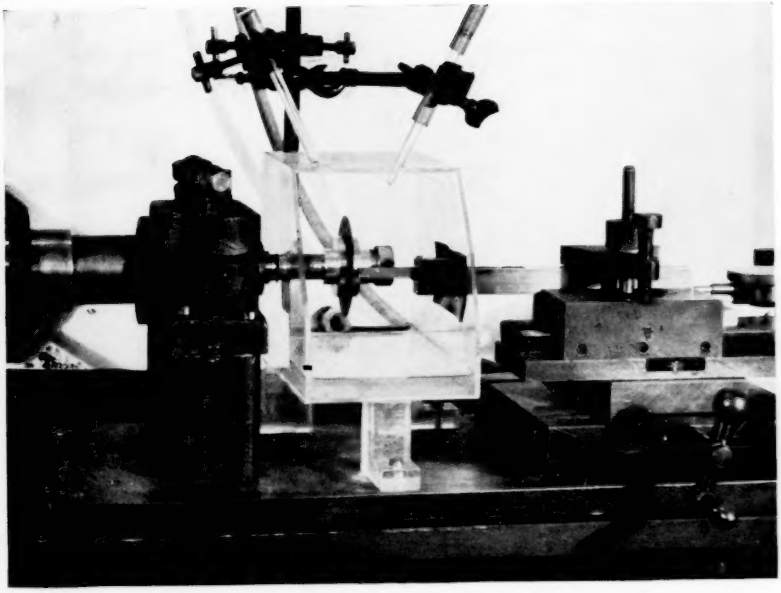

Fig. 2. Section-cutting machine; close-up view of cutting wheel.

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minute crystals have been oriented within the wire so that one direction in the majority of them is along one direction of the wire. In other words they have a "preferred orientation," and in respect of one axis they are behaving as a single crystal would behave. In the plane perpendicular to this direction the crystals are still oriented in a random manner. The inorganic crystals in bone show this kind of diffraction pattern, but it is the organic material which shows the pattern more usually associated with the description "fibre diagram." In this kind of material the degree of crystallinity is of a low order and the diffraction diagram only extends to a low value of 2θ, and usually consists of a few arcs or diffuse spots which indicate the presence in the specimen of regularly repeating units in certain directions rather than a truly three-dimensional crystal lattice. A review of the theory underlying the interpretation of X-ray fibre diagrams has been published recently by STOKES (1955) who gives some excellent examples of typical diagrams. In the same article is a brief introduction to the fundamental ideas of X-ray diffraction, and reference should be made to it if a fuller description than that given above is required.

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III. PREPARATION OF MATERIAL

III. 1. *Untreated bone*

In the case of all but the smallest bones it is necessary to reduce the specimen to the form of a plate or section of approximately 0·1 mm thickness. The reason for this is that, for X-rays of the wavelength normally used ($\lambda = 1{\cdot}54$ Å for Cu $k\alpha$ radiation), bone has a high absorption coefficient. It is desirable that the thickness of the specimen should be $1/\mu$ where μ is the linear absorption coefficient and the optimum thickness for bone is therefore approximately 0·1 mm. In the past it has been customary for chips of bone to be ground by hand to the required thickness, but a much more satisfactory method is to cut sections. If the bones under investigation are small, it is advisable to embed them in some plastic material but, with larger bones, sections may be cut without any previous preparation. The machine used for this purpose by the present authors is based on one developed by ATKINSON (1950) for cutting sections of teeth for optical microscope examination.

The sections are cut by means of a thin Carborundum Brand silicon carbide rubber-bonded grinding wheel which is rotated at high speed. ATKINSON found that, in order to cut sections of the order of 25 μ thick, it was advisable to use a speed of 12,000 revs. per minute with a 3-inch-diameter wheel; but although we have used a speed of 7000 revs. per minute for much of the work on X-ray diffraction, it is not necessary to use a speed greater than 3000 revs per minute in order to

cut sections of 0·1 mm thickness. The wheels (in thicknesses from 0·005 in. upwards) are made specially for this purpose by The Carborundum Co. Ltd. The thinnest wheels are rather fragile, but we have experienced very little difficulty in use if they are supported on the spindle by being clamped between discs of aluminium or brass with a thick washer of hard rubber on each side. The clamping discs and washers are of 2·4 in. diameter so that the maximum thickness of specimen must not be more than 0·25 in. This is easily achieved by cutting larger specimens into small pieces. The specimen is fixed by means of a suitable adhesive to a square perspex rod which is held in the tool holder of a compound slide taken from a small lathe. By means of these slides the specimen can be moved in two directions, one parallel to, and one perpendicular to, the plane of the cutting wheel. The thickness of the section is governed by reading a spring-loaded dial gauge attached to the cross-handle. The gauge reads to 0·0001 in.

The heavy spindle carrying the cutting wheel runs in a massive bearing which, together with the cross-slides, is bolted to a thick iron plate so that no relative motion of the parts can take place. The motor is mounted separately and a pulley and belt are used to drive the cutter.

If the machine is to be used for cutting sections of 50 μ or less, then it is necessary for all the parts, and especially the slides, to be made very carefully indeed; but standard slides from a small lathe are adequate for the sections needed for X-ray diffraction.

Water is used as a lubricant and for cooling the cutting wheel; two jets of water from a constant-level storage tank are allowed to play on the wheel, one on either side. The wheel and specimen are surrounded by a perspex box in which excess water collects to a depth of 1 in. before being piped to a sink. The sections are usually allowed to fall into the water, from which they can be recovered.

A machine, which uses screw slitting cutters, for cutting bone sections of thickness 80–150 μ, has recently been described by JOWSEY (1955). It has been pointed out, however, by the present authors (CLARK and IBALL, 1955), that the thin Carborundum wheels cause the loss of only 0·005 in. of material whereas the cutters used by Jowsey are 0·02 in. thick, and this thickness of specimen is lost for each section cut.

Sections of large bones can be cut without any preparation apart from making the blocks of bone small enough. For small bones, however, it is necessary to embed them in some suitable plastic. The use of methyl-methacrylate for embedding is well established, and it provides a suitable specimen for cutting bone sections. However, it is rather troublesome to prepare, and polymerization is very slow. The present authors have preferred a cold-setting polyester, "Marco Resin." There are only two constituents, the specially prepared resin mixture and a liquid hardener. These are quite stable and they only

need mixing immediately before use. Polymerization takes place at room temperature in a few hours. The polymerized material is not as hard as methyl-methacrylate, but for section cutting this is rather an advantage since the thin section does not break so easily. It is not necessary to dehydrate the bones when using this embedding plastic.*

III. 2. *Methods of treating bone specimens in order to improve the diffraction diagram*

The x-ray diagram from the whole bone is very unsatisfactory in many ways if it is desired to make accurate measurements of the lattice spacings. The rings are broad and only those rings which occur at low values of glancing angle θ (see II.1) are measurable. DAWSON (1946) showed that an improved pattern was obtained if the organic matter was removed by boiling the bones in water for 24 hr and then subjecting them to trypsin digestion at *p*H 8 for 4 to 5 days. BEAULIEU *et al.* (1950) pointed out, however, that the effect noticed by Dawson was produced by the boiling in water alone. This is the conclusion reached by the present authors. The improvement in the pattern produced by boiling is not easy to explain, since it is unlikely that any appreciable growth of crystals would take place at 100°C. Some change in the "content" of the crystal lattice could take place with the prolonged boiling, and it may be that the crystals in normal whole bone are to a slight extent "incomplete"; in other words there may be lattice defects which are partially rectified during the boiling. The effect on the x-ray diagram would be analogous to the change produced in the diagram of a metal when strains are removed by suitable heat treatment.

Although the effect of boiling is quite marked, a much greater improvement can be produced by calcining the bone. This can be done with a section, leaving the macroscopic shape unaltered if the heating is done carefully and gently. Alternatively the calcined bone can be powdered and treated as any other powder specimen. It may be that in the former case the organic matter is not completely removed but the heating is sufficient to produce a marked improvement in the diffraction pattern. In the latter case the best method is probably to mix the powdered bone with gum tragacanth and a little water. The paste can then be rolled into a rod (0·25 mm diameter) for insertion in the specimen holder of a standard powder x-ray camera. This kind of treatment produces the best x-ray diagrams of the inorganic constituent, and it is these which are almost indistinguishable from that of the mineral apatite. However, as many investigators have realized, it is no longer an x-ray diagram of bone but of something produced, by a

* The "Marco" embedding mixture was supplied by E. M. Cromwell & Co. Ltd., Galloway Road, Bishop's Stortford, Herts.

rather drastic treatment, from the inorganic chemical found in bone. There is no doubt that, as the earliest worker in this field pointed out (DE JONG, 1926), the crystals in calcined bone are much larger than those in normal bone; but it is still uncertain what other changes take place.

IV. RESULTS

It is not proposed to review all the hundreds of papers which have been published on this subject, but rather to attempt an assessment of the present position. There are five main divisions or topics under which the results obtained from X-ray diffraction can be discussed; the size of the inorganic crystallites, identification of the inorganic constituents, orientation of the inorganic crystallites, changes of structure due to age, and changes due to disease.

IV. 1. *The size of the crystals of the inorganic constituent*

It is not possible to see individual crystals in sections of bone under the ordinary microscope; and the effect of this small size, on X-ray diffraction diagrams, is to cause a broadening of the lines. This diffuseness is the reason why it is so difficult to provide an unequivocal decision on the exact nature of the crystals. The diffraction lines which occur at low values of the angle θ (see II.1) are diffuse but they are measurable, whereas the lines which should occur at higher values of θ are merged into the background. There is no doubt that most of this diffuseness is due to the small crystal size, but it is not certain that no other factor is involved. As pointed out earlier (III.2), it is possible to produce a very marked improvement in the sharpness of the lines by boiling bone samples. No evidence has been forthcoming that a mineral type of crystal will grow at a temperature of 100°C, although most writers on the structure of bone have assumed that bone crystals do grow under these conditions (CARLSTRÖM, 1955).

It is suggested that in normal bone the crystals are not complete but that the lattice, of an apatite type, has defects or sites not occupied by an atom or ion. The lattice may be "collapsed" to a very slight extent, and the effect will be to destroy the regularity of the repeat pattern of the crystal which will result in diffuse diffraction lines. Planes in the lattice of large spacing (low values of θ) will be least affected. At present this is speculation and, until experiments are devised to test it, one must assume that the diffuseness of the lines is due to the smallness of the crystals.

It is possible to calculate an approximate size for the crystals from measurements on the width of the high-angle diffraction lines and from measurements of the diffuse low-angle scattering. The values obtained vary, but the most recent estimates, obtained from the low-angle diffuse scattering, are 210×75 Å (ENGSTRÖM and FINEAN, 1953;

220 × 65 Å (CARLSTRÖM and FINEAN, 1954); 230 ± 20 Å for the length (CARLSTRÖM, 1955).

The electron microscope has been used to estimate the size of bone crystals, and the results do not agree with those given above. ROBINSON (1951, 1952) used bone (fresh or autoclaved) which was first thinly sliced and then treated in a Waring blender for ten minutes (ROBINSON and BISHOP, 1950). ROBINSON's estimate of the average crystal size was 500 × 250 × 100 Å, and a later estimate by ROBINSON and WATSON (1952, 1953) is 350–400 Å for length and width, with 25–50 Å for the thickness.

Electron microscopy seems to give a larger size than X-ray diffraction, and this would be expected if the suggestion put forward above, that the diffuseness of the X-ray diffraction lines is partly due to "strain" in the crystal lattices, is correct. On the other hand, estimates of size of this order of magnitude with the electron microscope must be treated with some reserve. The heating of the specimen by the electron beam could cause growth of the crystals, and in addition the calibration of the electron microscope cannot always be relied upon (BRADFORD and VANDERHOFF, 1955).

IV. 2. *Identification of the inorganic constituent*

This should normally be a relatively simple matter since the X-ray diffraction pattern of a crystal is unique, and it is only necessary to measure the lines of the pattern on an X-ray powder diagram and compare the results with those from a sample of the pure substance. The X-ray patterns from several thousand substances have been listed in a card index known as the A.S.T.M. Index, and identification of substances by this means is a standard and straightforward procedure. The difficulty with bone arises from the fact that the lines are so diffuse and that there are very few. If the bone is subjected to any treatment such as heating in order to improve the diffraction diagram, then it is not possible to be absolutely certain that no change has taken place.

The similarity between the structure of bone crystals and the apatite minerals was noticed from the earliest investigations with X-rays (DE JONG, 1926). Later ROSEBERRY, HASTINGS, and MORSE (1931) decided that the mineral dahllite (carbonate-apatite) most closely resembled the structure of bone, and they considered that secondary calcium phosphate ($CaHPO_4$) and $CaCO_3$ were not present as such in bone.

At a conference held at the beginning of 1950, ARMSTRONG (1950) attempted to collect and assess the various suggestions which have been put forward concerning the composition of the bone salts. However he could make no more definite a statement than that "the principal constituent of the bone salt is a calcium phosphate which has a space

structure resembling that of the earth apatites. This may be the material called α-tricalcium phosphate, or it may be hydroxyapatite." Of the carbonate present in bone he said that probably most of it is not directly built into the phosphate molecule, and that this equally applies to the citrate in bone. No considerable advance on this position is possible today.

At present there are three main schools of thought which claim that the inorganic constituent of bone is:

(i) α tricalcium phosphate together with free carbonate,
(ii) carbonate-apatite,
(iii) hydroxyapatite with carbonate adsorbed on the surface of the crystals.

The three leading supporters of each suggestion (i) DALLEMAGNE (1952), (ii) MCCONNELL (1952), and (iii) HENDRICKS (1952) reviewed the evidence for their own point of view at a conference in 1952. The evidence in all three cases is partly based on X-ray diffraction studies, but a great deal of chemical and physical-chemical research using many techniques has also been done. The X-ray diffraction evidence for each suggestion can be summarized as follows.

(i) DALLEMAGNE (BRASSEUR, DALLEMAGNE, and MELON, 1946) claims that the X-ray diffraction pattern of α tricalcium phosphate and the pattern of bone salt are identical. However, this is of a limited value since α tricalcium phosphate is isomorphous with hydroxyapatite and would have the same X-ray diffraction pattern in so far as the position of the lines is concerned. Small differences in the intensities would be present, but the pattern from bone salt is so lacking in definition that inaccuracies in measurement would swamp any real differences. It may be that, by using the more accurate techniques of electronic counters for measuring X-ray intensities, discrimination between the various isomorphous substances in this group will be possible. DALLEMAGNE (BRASSEUR, DALLEMAGNE, and MELON, 1946) believes that the bone salt in normal bone is α tricalcium phosphate and that, when the bone is heated, this is transformed into carbonate-apatite. The marked improvement in the sharpness of the diffraction lines after heating is attributed to the larger crystals of carbonate-apatite. Apart from chemical evidence, some more convincing data supporting Dallemagne's suggestion is provided by measurements of refractive indices. DALLEMAGNE (1945) and DALLEMAGNE and MELON (1944) found that the refractive indices of bone salts, and of dentine, were the same as that of a mixture of 90 per cent pure α tricalcium phosphate and 10 per cent calcium carbonate, viz. 1·590. After ignition of bone and dentine the refractive index changed to 1·649, which is the same as that of carbonate-apatite.

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Strong support on chemical evidence for the above has been provided by CARTIER (1948a, 1948b, 1951), and BARRAUD and CARTIER (1951). On the basis of these studies CARTIER came to definite conclusions regarding the composition of bone salt, and gave the following table:

	%
α tricalcium phosphate	74·6
calcium carbonate	10·4
calcium citrate	2·0
trimagnesium phosphate	0·93
magnesium carbonate	1·03
disodium phosphate	2·46
Ca^{++}, PO_4^{---} } Protein	8·76

The last item, 8·76 per cent, remains in the bone when it is treated with acid, and according to CARTIER it corresponds to a combination of the radicals Ca^{++} and PO_4^{---} with the organic matrix of bone.

Although the conclusions of DALLEMAGNE and CARTIER have been criticized, e.g. BRANDERBERGER and SCHINZ (1948), SOBEL (1952), it can be stated that the evidence from X-ray diffraction is not inconsistent with them.

(ii) One of the earliest suggestions that the carbonate was included in the apatite-like lattice was put forward by ROSEBERRY, HASTINGS, and MORSE (1931) who, as stated above, decided that the bone diffraction pattern resembled dahllite more closely than any other apatite. BOGERT and HASTINGS (1931) supported this, and GRUNER, MCCONNELL and ARMSTRONG (1937) showed how the carbon atoms could take the place of calcium and phosphorous atoms in the apatite lattice. A detailed discussion on substitutions in apatites is given by MCCONNELL (1938). These authors seem to agree that the main part of bone salt can be represented by a substance having an apatite-like lattice with a formula $(OH)_2Ca_6[\{(P, C)O_4\}_6 (Ca, C)_4]$, i.e. the crystals have the same *structure* as hydroxyapatite, but with some of the Ca and P sites in the lattice occupied by C atoms. There is, however, considerable doubt whether so-called carbonate-apatite minerals are really single phases or very intimate mixtures of microcrystalline fluorapatites and extremely small particles of $CaCO_3$ (CARLSTRÖM, 1955).

(iii) KLEMENT (1929) and KLEMENT and TRÖMEL (1932) suggested that the X-ray diffraction pattern of bone salt most closely resembled that of hydroxyapatite. More accurate measurements (CARLSTRÖM, 1955) indicate that the lattice spacings of bone salt agree better with those of synthetic hydroxyapatite than with any other of the substances proposed.

The surface area of the inorganic crystallites is so large that adsorption on their surfaces of carbonate and other ions could readily take place. HENDRICKS and HILL (1950, 1951) considered this aspect very

carefully, and came to the conclusion that the bone crystals consisted of a neutral calcium phosphate, probably hydroxyapatite, with carbonate, citrate, sodium, and magnesium ions adsorbed on their surfaces. The actual surface area involved has been estimated to be more than 100 acres in an adult man (NEUMANN and NEUMANN, 1953).

TABLE 1

Interplanar Spacings observed by Several Investigators for Bone Sections and Powdered Bone

Bone sections								*Powdered bone*		
A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
—	—	—	—	—	—	—	—	—	17·11	—
—	—	—	11·5	—	—	—	—	—	—	—
—	—	8·29	—	8·30	—	—	—	—	8·29	—
—	—	—	8·02	—	—	—	—	—	—	—
—	—	5·31	—	5·30	—	—	5·21	—	—	—
—	—	4·90	4·96	—	—	—	—	—	4·92	—
—	—	4·12	—	4·20	—	—	—	—	—	—
—	—	3·85	3·67	—	3·77	3·86	3·85	—	3·96	—
3·5	3·38	3·50	—	3·44	3·38	3·42	3·42	—	3·43	3·41
3·35	—	—	3·33	—	—	—	—	3·35	—	—
—	—	3·22	—	3·17	—	—	—	—	—	—
—	3·07	3·10	—	—	3·08	3·13	3·10	—	3·12	3·11
—	—	—	3·00	—	2·99	—	—	3·02	—	—
2·8	—	2·84	—	2·87	—	2·77	2·76	—	2·82	2·76
—	2·70	2·67	2·71	2·69	—	—	—	2·72	—	—
—	—	2·60	—	—	2·62	2·59	2·63	—	2·58	—
—	—	2·37	—	—	—	—	—	—	—	—
—	—	2·30	—	—	—	—	—	—	—	—
2·2	2·25	—	2·23	—	2·24	2·24	2·26	2·25	2·26	2·25
—	—	2·08	—	—	—	—	—	—	2·10	—
—	—	2·00	—	—	2·04	2·05	2·05	—	—	—
—	1·92	—	1·91	—	1·92	1·91	1·94	1·93	1·88	1·93
—	—	1·85	—	—	1·85	1·85	1·84	1·83	—	1·83
—	1·81	—	1·80	—	1·82	—	—	—	—	—
—	—	—	—	—	—	—	—	—	1·76	—
1·7	—	1·71	—	—	1·70	1·70	1·72	1·71	1·72	1·71
—	—	1·63	—	—	—	—	—	—	1·68	—
1·6	—	1·58	—	—	—	—	—	—	—	—
1·4	—	—	—	—	—	—	1·45	1·44	—	1·45
—	—	—	—	—	—	—	—	1·30	—	—
—	—	—	—	—	—	—	1·25	1·21	—	1·23
—	—	—	—	—	—	—	1·11	1·10	—	1·11
—	—	—	—	—	—	—	1·02	1·02	—	1·02

A = CLARK J. H. (1931)
B = CLARK G. L. and MEGUDICH (1934)
C = REED and REED (1942a)
D = HENNY and SPIEGEL-ADOLF (1947)
E = BELL *et al.* (1947)
F = ENGSTRÖM and ZETTERSTRÖM (1950)
G = ENGSTRÖM and ZETTERSTRÖM (1951)
H = Present authors
I = ROSEBERRY *et al.* (1931)
J = REED and REED (1942a)
K = Present authors

It seems most probable that the inorganic constituent of bone is not a single chemical substance of fixed composition, but that most of it consists of crystals of a calcium phosphate closely resembling hydroxyapatite and/or α tricalcium phosphate, both in structure and composition. These crystals will differ slightly in composition from time to time in the same bone, and from one bone to another. There seems to be no other explanation of the differences of spacings found by different investigators, since these differences are larger than the experimental errors. A comparison is given in Table 1 of the lattice spacings for normal bone, both as sections and powdered specimens, found by several investigators. Even if allowances are made for the variation of definition of the lines on the photographs, the differences shown are greater than expected. A careful investigation with one of the recently developed precision x-ray diffractometers using Geiger-Müller counters would probably make possible a definite conclusion on these points.

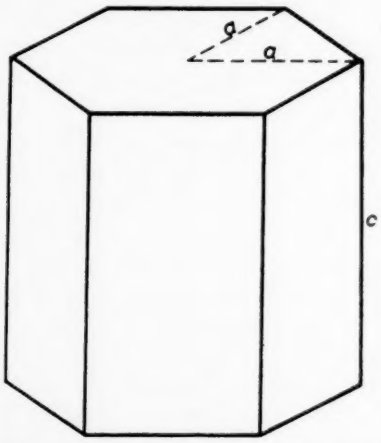

Fig. 3. Diagram of hexagonal crystal showing unit-cell axes.

The spacings given in columns H and K of the above table were found to be consistent with a hexagonal unit cell of dimensions $a = 9{\cdot}37$ Å, $c = 6{\cdot}88$ Å. The a axis is the radius of the hexagonal base of the hexagonal prism and c is the height.

Values for the unit cell dimensions of bone found by other investigators are listed by CARLSTRÖM (1955) and vary from 9·29 to 9·50 Å for a and from 6·87 to 6·96 Å for c. Precision measurements by CARLSTRÖM on bone powder heated to 900°C for 2 hr gave: $a = 9{\cdot}421 \pm 0{\cdot}002$ Å, $c = 6{\cdot}882 \pm 0{\cdot}002$ Å; and he found the following values for tooth enamel treated in the same way, $a = 9{\cdot}429 \pm 0{\cdot}002$ Å, $c = 6{\cdot}884 \pm 0{\cdot}002$ Å.

The crystallites in bone appear to be tabular in form as stated above (IV.1) with the long axis of the crystallite parallel to the *c* or vertical axis of the hexagonal prism which forms the unit cell of the crystal lattice.

IV. 3. *The orientation of the inorganic crystallites*

IV. 3. 1. *General*

There is considerable confusion and some direct contradiction in the literature regarding the way in which the submicroscopic inorganic crystals in bone are aligned with reference to the shape of the bones or to the direction of the organic fibres in the bone. Some of the confusion is undoubtedly due to the different methods of preparing specimens and to different techniques of taking x-ray diffraction photographs.

As regards the preparation of material for studies of orientation, it is essential to have some method of cutting thin sections with means of orienting the bone precisely with reference to the cutting tool. If the specimens are small it is necessary to embed them in some transparent medium before attempting to prepare sections. The technique employed by the present authors and described in III.1 has proved very satisfactory over several years.

The essential point in connection with the x-ray diffraction technique is that the beam of x-rays should be very small in cross-section, or in other words the area of specimen investigated must be small. *Extremely* narrow beams are not always necessary, but in general the beam should not be larger than 0·1 mm diameter at the specimen. With small beams of 0·1 mm or less, it is necessary to use a miniature x-ray diffraction camera, and the one described by CHESLEY (1947) is very suitable for this purpose. The camera used by the present authors was specially constructed on the basis of the CHESLEY camera. It had lead collimators of 0·1 mm, and specimen-to-film distances of 0·5, 1·5, 2·0, and 2·5 cm respectively. Such cameras are available commercially now, and can be fitted with lead-glass collimators giving x-ray beams of 0·1 mm, 0·05 mm, or less. With the recent development of microfocus x-ray tubes, miniature cameras can be employed to great advantage with a considerable reduction in exposure time. The x-ray diffraction photograph obtained from a section of a long bone such as a femur shows the pattern known as a "fibre" pattern which, as is explained in II.3, is intermediate between those given by a single crystal rotated about a principal axis of the crystal lattice and a polycrystalline powder in which the crystallites are oriented randomly. This "fibre" diagram from long bones is due to the fact that the majority of the submicroscopic crystallites are oriented with the *c* axis of the hexagonal lattice parallel to the length of the bone. Such a photograph is shown in Fig. 4. This orientation is shown most clearly

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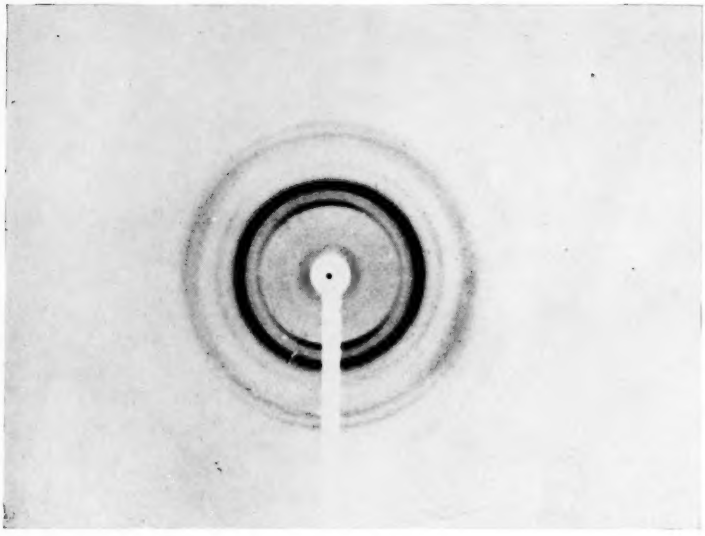

Fig. 4. Typical x-ray diffraction photograph of longitudinal section from the femur of a rat three months old. Mo$K\alpha$ radiation.

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by the inner ring on the photograph. If the crystallites are oriented so that the basal plane (002) of the hexagonal prism is almost horizontal, then they will be in a position to reflect more X-rays to the vertical segments of the (002) ring on the film than to other parts. This is what has happened in the case of Fig. 4. The other rings do not show this effect so clearly in the reproduction, but in the original photographs many of the rings can be seen as "arcs" only and not complete rings of uniform density. Fig. 5 is a drawing of a typical photograph which shows the effect actually observed.

Some of the early workers who investigated pieces of whole bone came to the conclusion that the inorganic crystals were not oriented in any preferential manner, e.g. HENSCHEN, STRAUMANN, and BUCHER

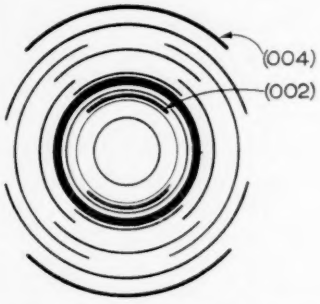

Fig. 5. Diagram of a typical X-ray diffraction photograph of a normal adult bone. The appearance of incomplete rings indicates orientation of the crystallites in certain preferred directions.

(1932). However, there can be no doubt now that in long bones the inorganic crystallites are arranged preferentially with the *c* axis of the unit cell parallel to the direction of the collagen fibres (ENGSTRÖM, ENGFELDT, and ZETTERSTRÖM, 1952; KOISI, 1941; JENSEN and MOLLER, 1948; LAMARQUE, 1943; and REED and REED, 1942). The electron-microscope studies by ROBINSON and his colleagues confirm the finding of FINEAN and ENGSTRÖM (1953) that the long axis of the *crystallites* is, more often than not, parallel to the collagen fibres (ROBINSON, 1951; ROBINSON and WATSON, 1952).

Several groups of workers have attempted to find out if mechanical forces acting on the bones have been responsible for the preferred orientation of the inorganic crystallites. In this connection REED and REED (1942) compared the diffraction patterns from bones which had been subjected to muscular activity (tibiae) and those which had not (skull bones). In both cases the diffraction patterns showed the usual arcing of the diffraction rings, and they therefore concluded that the preferential orientation of the crystals in bone is not due to muscular activity. LAMARQUE (1943) also investigated this problem, and he did

not find any direct relation between the mechanical role played by each part of the bone and the orientation of the crystals in that part.

On the other hand, the opposite conclusion was reached by CAGLIOTTI and GIGANTE (1936), and by HENNY and SPIEGEL-ADOLF (1945, 1947). CAGLIOTTI and GIGANTE found that in the long bones, i.e. the weight-bearing bones, the orientation was clearer at an earlier stage of development than in the parietal bones which had merely the function of protection. To study this problem HENNY and SPIEGEL-ADOLF used two kinds of fish ribs which were approximately at right angles to each other. They found that only one of these sets of ribs showed preferential orientation of the inorganic crystals, and they concluded that orientation of the crystals in bone must be related to the functional role of the bone.

This question has been settled in a convincing manner by ENGSTRÖM and AMPRINO (1950). They succeeded in completely immobilizing one of the legs of a dog for a time which was sufficiently long for all of the material in the compacta of the bones to have been laid down during the period of immobilization. When x-ray diffraction patterns of bone from the normal and inert limbs were compared they were found to be identical. It therefore appears that the ultrastructure of bone is independent of the forces acting on the bone.

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IV. 3. 2. *Effect of age on orientation*

Very few results have been reported regarding the changes which take place, or do not take place, in orientation as the age of the bone changes. ENGSTRÖM and ZETTERSTRÖM (1951) stated that they observed no changes in either lattice spacings or orientation with age.

In order to investigate this question of orientation more systematically, the present authors examined the femora of a pure strain of white Wistar rats.*

A large number of rats of different ages was obtained by breeding from one or two pairs. Immediately after each of the rats was killed its two femora were removed and cleaned of adhering tissues. The ages of animals ranged from birth to 475 days. It was found that no change could be detected in the lattice spacings of the different bones, but there was a marked change in the orientation.

Longitudinal sections of the femur were cut as shown in Fig. 6, from the midsection in the case of older bones and from the whole bone in the case of very young ones.

It was found that with rats up to approximately 3 days from birth no preferred orientation of the crystallites could be detected. After 3 days slight orientation appeared, and this increased until at about

* We are indebted to Professor A. HADDOW of the Chester Beatty Research Institute for a supply of these rats.

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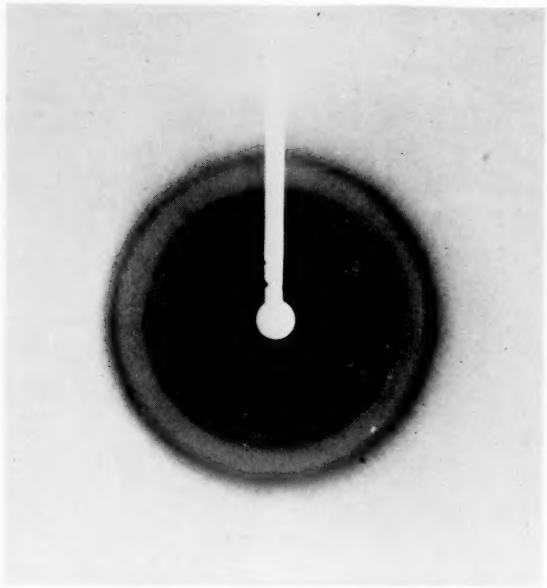
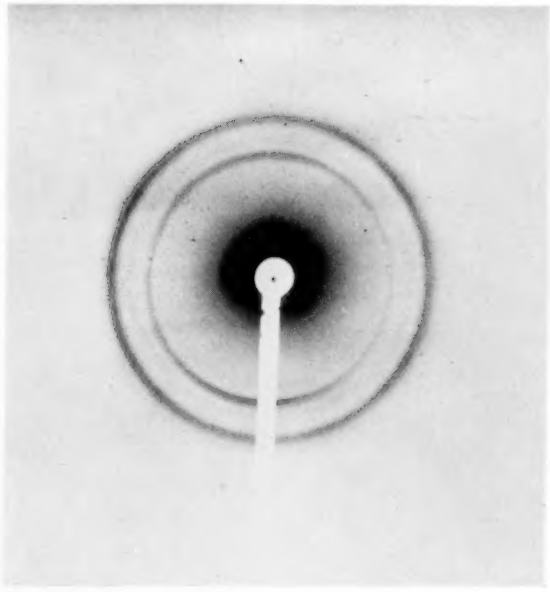


(*a*)



(*b*)

Fig. 7. X-ray diffraction photographs from longitudinal sections from mid-shafts of rat femora. (*a*) 3 days old, (*b*) 15½ months old. Note the incomplete (002) ring in (*b*) showing orientation; whereas in (*a*) the rings are complete indicating no orientation. There is an additional diffuse ring in (*a*).

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13 days the orientation was as marked as in adult bone. In the very young bones an extra diffuse ring appeared inside the (002) ring of the inorganic crystal pattern. Fig. 7 shows the change which occurs.

Following these results a further investigation was carried out to see whether bones from different species of animal would behave in the same way. The femur of newborn golden hamsters gave a similar result. No orientation at birth was observed, but marked orientation appeared afterwards. Guinea-pigs on the other hand were different. The femur showed orientation at birth. Only a few specimens of the hamster and guinea-pig were examined, but an extensive investigation was carried out on human bones.

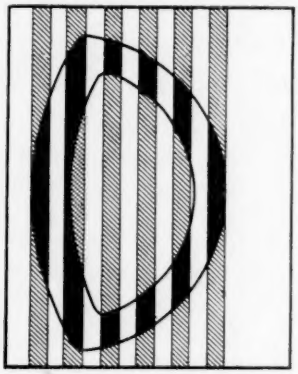

Fig. 6. Outline of a cross-section of a femur showing the way in which longitudinal sections were cut.

Human bones, ranging in age from newborn to adult, and in addition human foetal bones, from approximately two months' intrauterine development, were studied in the same way as were the rat bones described above. As far as lattice spacings were concerned no difference was detected between bones of different ages, nor was there any difference between human bones and rat bones. All these results refer to the femur.

Newborn human femora showed marked orientation of the crystallites so the examination of foetal bones was carried out to find out whether there was any stage in their development at which the crystallites were randomly oriented. (The ages of the foetal specimens were estimated from the probable date of conception and the C.R. (crown-rump) length of the foetus.) The number of specimens was necessarily much smaller than with rats. They fell into the following age groups: (*a*) 2 of about 2 months' development, (*b*) 3 of 3 months, (*c*) 1 of 3·5 months, (*d*) 3 of 4 months, (*e*) 2 of 4·5 months, and (*f*) 3 of 5 months.

With these foetal bones the same kind of change in the orientation

was observed. Up to about 4 months' development no appreciable orientation could be detected, at 4·5 months specimens showed slight "arcing" of the (002) ring on the photographs, and in the case of those of 5 months' development the orientation was quite marked. In fact the degree of orientation at 5 months' intrauterine development was almost the same as in an adult bone.

At 7 to 8 weeks' development the bones are soft, and it is only a very small portion of the midshaft of the femur which is ossified, the rest of the bone being modelled in cartilage. The diffraction pattern of a human femur of that age is very similar to that of a rat femur 3 days after birth. It shows no evidence of inorganic crystal orientation, and it has the diffuse inner ring which corresponds to a lattice spacing, or repeat distance, of 4 to 5 Å.

IV. 3. 3. *Variation of orientation along the length of the bone*

Several investigators, e.g. CLARK and MRGUDICH (1934), and BELL, CHAMBERS and DAWSON (1947), have pointed out that the degree of orientation of the inorganic crystallites appears to vary at different positions along the length of long bones. No details were given in their papers, however, particularly about the relative length of the bones in which orientation existed. The present authors therefore decided to examine longitudinal sections of bone to clear up a number of points in this connection.

Adult rat bones were first studied. X-ray diffraction photographs were taken at different points along the length of each longitudinal section, and it was found that near the ends of the bones (the metaphyses) no orientation was observed, but as the midshaft was approached the orientation appeared. With rat femora of all ages in which orientation appeared at all it was found in at least one-third of the length of the bone, this being the middle portion of course. Typical examples were as follows: (*a*) 9 days old, total length 7·1 mm, orientation present over the middle 3 mm; (*b*) 15 days old, total length 15 mm, orientation present over 6 mm. There did not appear to be any *gradual* lessening of the degree of orientation as the area examined (0·1 mm diameter at 0·5 mm intervals) moved from the middle towards the epiphyses. Two areas only 0·5 mm apart would be found, one showing the same orientation as the middle of the bone and the other no orientation at all. A corresponding examination on human bones gave the same result.

In addition to the above investigation on bones which show orientation a similar study was made at different points along the length of sections of bones which did not show orientation in the middle portion of the shaft. For example, x-ray diffraction photographs were taken at 0·5 mm intervals along the length of sections of a bone from a human

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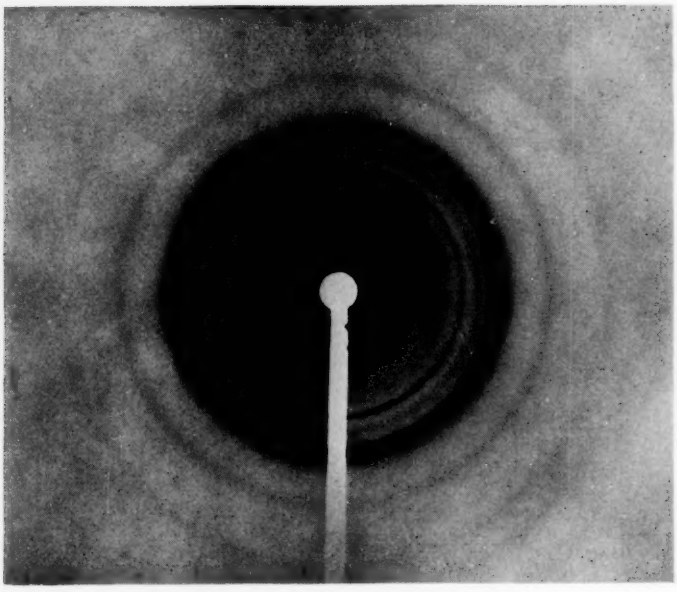

(*a*)

(*b*)

Fig. 8. X-ray diffraction photographs from midshaft of femur of rat 4 months old. (*a*) cross-section, (*b*) longitudinal section. Note that in (*a*) the (002) ring is relatively weaker than in (*b*), but it shows that orientation is present.

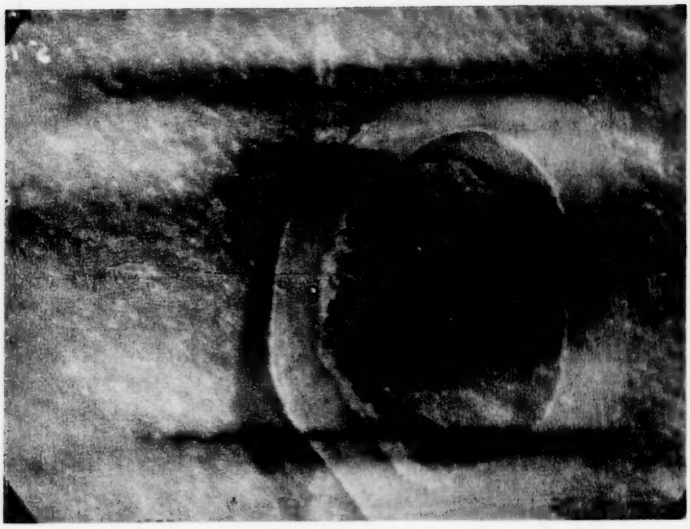

Fig. 9. Photomicrograph of a cross-section of the femur of a rat 4 months old, showing the spot of light which indicates the area to be irradiated by x-rays.

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foetus of 3 months' development. None of the photographs showed any appreciable orientation.

IV. 3. 4. *Cross-sections of long bones*

Among the relatively few papers which mention X-ray diffraction studies of cross-sections are those of CLARK (1931), CLARK and MEGUDICH (1934), HENNY and SPIEGEL-ADOLF (1945), and ENGSTRÖM and ZETTERSTRÖM (1951). All these authors state that no orientation was observed in cross-sections of bones, but no details are given; the reference is usually a single sentence such as "In cross-sections no orientation was observed" (ENGSTRÖM and ZETTERSTRÖM, 1951). None of them apparently used a section-cutting machine and, since the preparation of satisfactory cross-sections without such a machine is extremely difficult, it is perhaps not surprising that little attention has been paid to them.

The present authors have made a systematic investigation of cross-sections (CLARK and IBALL, 1954) prepared with the aid of the section-cutting machine described above (III.1), and the results contradict previous statements in regard to orientation. Fig. 8 shows for comparison diffraction photographs of a longitudinal section and of a cross-section of the same bone. From these photographs it was clear that (1) the same diffraction rings appear on both and they are in the same positions, (2) the relative intensities of the rings are not the same on both. In particular the (002) ring is much less dense in relation to the uniformly dense diffuse ring; and so also, but less noticeably, are the (202), (113), and (004) rings. (3) The (002) ring shows "arcing" on the cross-section photograph as well as on the longitudinal section one. This indicates that, of those crystallites which have their *c* axes perpendicular to the length of the bone, a majority are arranged parallel to *one* direction in the cross-section.

In the case of longitudinal sections the direction of orientation of the inorganic crystallites is always related to the collagen fibre axis which is parallel to the length of the bone. With cross-sections there is no obvious direction to which the direction of orientation could be related. The sections were mounted on the X-ray camera, and a strong beam of light shone through the collimator. A low-power photomicrograph was taken before each X-ray exposure so that the position of the X-ray beam with reference to the specimen was known (see Fig. 9). In addition, the position of the specimen with relation to the camera and therefore with reference to the film was known. Consequently it was possible to determine how the direction of preferred orientation as shown on the film was related to the shape of a particular specimen. The results of a large series of photographs at different points around the cross-sections show quite clearly that the crystallites are

preferentially oriented with their c axes approximately tangential to the surface of the bone. Fig. 10 is one example of the results obtained.

This diagram is a tracing from a photomicrograph of the cross-section of the femur of a rat 10 days old. The points on the section where x-ray diffraction photographs were taken are marked with small circles. Radial lines are drawn, from each of these points, perpendicular to the tangent to the surface near to the points. Then the direction of orientation (of the c axis of the crystals) is indicated by short lines at

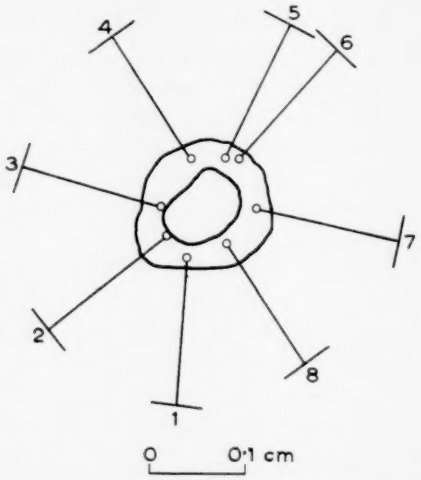

Fig. 10. Outline of a cross-section of the femur of a rat 10 days old showing the points at which the diffraction photographs were taken and the corresponding directions of preferred orientation of the c axes of the crystallites.

the end of the radial lines. The angles between the direction of orientation and the corresponding radial line in this particular section varied between 86° and 90° for positions marked 1 to 8. Sections from rat bones aged 10 days to 10 months were examined in this way and in every section orientation similar to the above was found.

IV. 4. *Changes in structure due to disease*

Considerably more work has been done on rachitic bones than on bones affected by any other pathological condition, but almost every disease of bone has been examined to try and find a correlation between change of structure and pathological change. Each disease will be reviewed separately.

IV. 4. 1. *Rickets*

Shaumann (1932) reported that with rachitic bones the diffraction lines due to the organic matter were of greater relative intensity than

with normal bones. This result has not been confirmed. SAUPE (1934) also commented on the pattern from the organic component of bone, and he suggested that the broad diffraction lines which he observed were due to extra water in the rachitic bones. CLARK and MRGUDICH (1934) claimed that the degree of orientation in rachitic bones was appreciably less than in normal bones. Results which confirmed this disorientation of the inorganic crystallites were reported by REED and REED (1942a, 1942b), and the same authors in a later paper (1945) stated that they had improved the technique and had found that the difference between normal and rachitic bones was more definite than their earlier results had indicated. In their experiments REED and REED used the cortices of the tibial midshafts of rats. They claimed that the degree of orientation was significantly less than normal in the bones of rats on a rachitogenic diet, and that the degree of orientation increased when the animals were given vitamin D. However, BELL, CHAMBERS, and DAWSON (1947) carried out a series of mechanical, chemical, and X-ray diffraction tests on rat bones, and concluded that there was no disturbance of the fundamental plan of ossification in rachitic bones. They could discover no difference between the degree of orientation of normal and rachitic bones.

ENGSTRÖM and ZETTERSTRÖM (1951) investigated, by means of X-ray diffraction, the bones of a dog which had been on a rachitic diet, when 2 months old, for 5 weeks, and which developed severe rickets. They could find no evidence of a change in the diffraction pattern.

The present authors examined a large series of femora of rats which had been given different diets: (i) controls, fed on standard rat cake and water, (ii) rats fed on the STEENBOCK and BLACK (1925) rachitogenic diet, (iii) rats fed on the STEENBOCK and BLACK diet with the addition of 5 per cent w/w of lysine-hydrochloride. These animals were part of an investigation by Dr. JOAN PERRY who carried out many tests of different kinds (PERRY, 1954). We are indebted to Dr. PERRY for providing the femora for the X-ray diffraction studies. The X-ray diffraction patterns from rachitic bones were identical with those from normal bones. That the animals fed with a rachitogenic diet had severe rickets was proved by radiographic examination and by breaking tests (PERRY, 1954). It is difficult to understand how such conflicting results as those of REED and REED on the one hand and those of BELL, CHAMBERS and DAWSON, ENGSTRÖM, ZETTERSTRÖM, and ourselves, on the other, can be reconciled.

IV. 4. 2. *Other diseases*

The same kind of conflicting evidence runs through the papers on other bone diseases; one set of investigators finds that the degree of

orientation is different in diseased bones from that in normal bones, while the other set find no such difference. Some examples are as follows:

Hyperparathyroidism. REYNOLDS, CORRIGAN, and HAYDEN (1938) found a difference between the X-ray pattern of normal bones and those of animals suffering from hyperparathyroidism. On the other hand BRANDENBERGER and SCHINZ (1945) and ENGSTRÖM and ENGFELDT (1951) could detect no difference between normal and diseased bones.

Paget's disease. REYNOLDS, CORRIGAN, and HAYDEN (1938) did not detect any difference with this disease, whereas ENGFELDT *et al.* (1952) claimed that the degree of orientation of the inorganic crystallites was considerably less in bones from animals suffering from PAGET's disease than in normal bones.

Osteomyelitis. CLARK, BUCHER, and LORENZ (1931) found that the diffraction lines due to the inorganic component were weaker, in relation to the intensity of the lines due to the organic component, in bones with osteomyelitis. BRANDENBERGER and SCHINZ (1945) could find no such effect.

Bone cancer. BRANDENBERGER and SCHINZ (1945) compared the diffraction pattern of cancerous bone with normal bone and could detect no difference, thus contradicting an earlier finding of CLARK, BUCHER, and LORENTZ (1931). CARLSTRÖM and ENGFELDT (1954) found the diffraction pattern from untreated osteogenic sarcoma to be identical with those from normal bones.

Excess fluorine diet. Again BRANDENBERGER and SCHINZ (1945) could find no difference in the orientation of bone crystals when rats were given an excess of fluorine in their diet. REYNOLDS, CORRIGAN, HAYDEN, MACY, and HUNSCHER (1938) had reported that this diet produced bones in which there was almost no orientation of the inorganic crystals.

It is unfortunate that so many investigators have not given sufficient detail of the way in which their specimens have been prepared and the exact site examined. There is more variation in normal bones than the earlier workers realized, and it is doubtful whether any of the claims to have detected a difference between diseased and normal bone could be substantiated.

V. CONCLUSIONS

The application of X-ray crystal-analysis techniques to the study of bones has been carried out since 1926, but the results obtained have been rather disappointing. The reason for this seems to lie in the variable nature of the material rather than in the limitations of the method. The method has given information regarding the size of the inorganic crystallites, but has failed so far to produce unequivocal

evidence on the exact composition of the crystals. The present authors wish to suggest that the crystals in untreated bone do not have a *fixed* composition, and may not have all the lattice sites filled at any given time. Treating the bone gently, e.g. by boiling, causes the crystals to become more "perfect," i.e. the lattice sites are more completely occupied, with consequently reduced strain and better defined x-ray diffraction lines. More violent treatment, e.g. calcining, has the further effect of causing the crystallites to grow.

From the evidence produced by x-ray diffraction, there appears to be little to choose between hydroxyapatite and α tricalcium phosphate as representing the most probable composition of the normal bone crystals, with perhaps a slight preference for hydroxyapatite.

Orientation of the crystallites is marked in long bones, e.g. the femur. Most of the hexagonal crystallites are oriented with their c (or six-fold) axis nearly parallel to the collagen fibre axis, but it appears that some crystallites are arranged with their c axes perpendicular to the fibre axis, and these tend to lie with their c axes tangential to the surface of of the bones.

At a very early stage in the growth of long bones no orientation of the inorganic crystals can be detected. This stage may be observed in some animals (e.g. rats) after birth, but in human bones it is necessary to examine foetal bones in order to observe the stage in growth at which orientation appears.

It is advisable, and in some cases absolutely necessary, to have some means of cutting thin sections of bone if x-ray crystal analysis of untreated bone is to yield results free from confusion. This is especially true of orientation studies. In addition it is necessary to use small x-ray beams.

Many different kinds of diseased bones have been examined, but there does not appear to be a single case where it can be stated categorically that x-ray crystal analysis reveals a difference in structure between a diseased and a normal bone.

A fruitful field for research in the future would be to investigate the relationship between the inorganic crystallites and the organic material of bone. This would involve using x-ray crystal analysis, electron-micrography, and other techniques *on the same material*.

The collagen of bone *appears* to be the same kind of material as the collagen of connective tissue, but no explanation has been forthcoming up to now of the fact that, in bone, ossification takes place. The organic matrix which is largely composed of mucopolysaccharides probably plays an important role in this connection. If the process of calcification was thoroughly understood, it would then seem probable that the obvious differences in behaviour between diseased and normal bone could be related to their structure.

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MUSCLE STRUCTURE AND THEORIES OF CONTRACTION

A. F. Huxley

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MUSCLE STRUCTURE AND THEORIES OF CONTRACTION

A. F. Huxley

Introduction

It has seemed natural to all who have studied voluntary muscle with the microscope to suppose that the striations are an important clue to the mechanism of contraction. Until the early years of this century, they were almost the only clue, apart from the process of shortening or tension development itself, and interest was accordingly centred on the changes that the striations undergo during contraction. But no coherent theory of contraction emerged from these studies; perhaps it is for this reason that the hard-won knowledge of muscle structure fell into neglect as rapid progress began to be made in muscle chemistry and in the energetics of contraction.

Up to the outbreak of the second war, these branches of muscle physiology were able to proceed successfully without reference to the details of structure. It appears now that the discoveries that were made in muscle biochemistry up to 1939 were relevant to recovery processes rather than to the contraction mechanism itself. This is not to belittle their importance: from the point of view of muscle physiology they led to the discovery of ATP and to the idea that this substance is the immediate source of energy for contraction, while in a broader context they laid the foundations of our understanding of the glycolytic cycle and of the ways in which energy is made available for the activities of all kinds of cells. It does however mean that these discoveries had little immediate relevance to the problem of how the contraction itself is brought about; conversely the characteristic features of muscle structure were irrelevant to the processes that were being elucidated. This position was brought to an end by the discovery of the interactions of "myosin" and ATP, and of the composite nature of "myosin." It could be said that muscle chemistry had then become ripe for integration with information about structure, but in the meantime the knowledge that had been gained by the nineteenth-century microscopists had been largely forgotten. This is illustrated for example by comparing the brief accounts of the striations, and of their changes during contraction, that are to be found in modern text-books, with the full and accurate description in say the 1888 edition of Michael Foster's text-book.

The chemistry of the muscle proteins did indeed retain some contact

with microscopical information; for example NOLL and WEBER (1934) showed that the birefringence of the *A* bands could be accounted for by the birefringence of "myosin" threads. This contact seems to have been lost at the time when it might have been most fruitful; there was no attempt to locate myosin proper and actin with respect to the striations until more than a decade after the separation of these proteins.

Knowledge of the mechanics and heat production of muscle reached a stage at which they were ready to link up with other lines of information on the contractile process in 1938, when A. V. HILL published his well-known analysis of the relations between tension, speed of shortening, and heat production. However, in spite of HILL's repeated calls for chemical studies to parallel his analysis of the time course of energy liberation, this work remained isolated until the recent demonstration by FLECKENSTEIN *et al.* (1954) and MOMMAERTS (1954) that the amounts of ATP and creatine phosphate in a muscle do not fall during the contraction phase of a twitch.

As regards the processes by which the contractile mechanism is "turned on," it was clear by the end of the war (KUFFLER, 1946; KATZ, 1950) that the reduction of the resting potential which occurs on excitation is the normal stimulus for contraction, and a number of physical changes were known to begin during the "latent period" between excitation and the development of tension or shortening (see pp. 300–304).

Although the basic facts concerning the striations, which had been known for 80–100 years, were unduly neglected at this time, it must be admitted that our knowledge of muscle structure had not made much progress since the turn of the century, and in relation to the available techniques it was less advanced than other branches of muscle physiology. Even the application of the electron microscope did little at first beyond confirming (and restoring to respectability) the picture that had been handed down by the nineteenth-century microscopists; indeed, several of the new conclusions that were drawn from the early work with the electron microscope have since turned out to be unfounded. But in the last few years a number of important points about the striations themselves, and about the spatial distribution of the structural proteins, have come out, and as a result it is now possible to make statements about the intimate structure of muscle which are sufficiently detailed to have a bearing on the interpretation of the mechanical, thermal and chemical events. It remains for the future to decide how far the views that I am adopting here about the structure of muscle are correct, but I do not think that they are any more likely to be in error than are the current statements of its mechanical and chemical behaviour.

INTRODUCTION

Up to now, the theories of muscle contraction that have been put forward have often been based on only a single aspect of muscular activity. The lactic-acid theory, which might be classified as primarily chemical, did indeed take account of what was then known of heat production and work done by the muscle. But ASTBURY's (1947) theory was purely structural and RAMSEY's (1944) takes account of little except the energetics of contraction, while most of the other recent theories are chemical and make no attempt to accommodate either the relationships which HILL found to govern the release of energy as heat and work, or the known features of structure. POLISSAR's theory (1952a,b,c,d) is very comprehensive as regards mechanical and thermal data, and could probably accommodate the known chemical events, but it takes no account of muscle structure and it does not fit with some of the important facts about heat production (WILKIE, 1954).

These theories have done good service by suggesting lines for further work and by bringing together observations from more or less diverse fields. Their onesidedness has been a natural consequence of the fact that the different branches of muscle physiology had hardly made contact with one another; this did mean however that there was little chance that a generally satisfying theory would be produced. But this is no longer the position: it should by this stage be possible to produce hypotheses which try to account simultaneously for the mechanical, thermal, chemical and structural changes which are known to occur during contraction. Later in this article I shall try to make good this statement by putting forward a hypothesis which does this to some extent. It achieves a fair agreement with observation in several respects, but I do not suppose it is by any means the only scheme that could be devised at the present day with an equal degree of success.

Much of the new evidence on muscle structure has been recently reviewed (PERRY, 1955; HANSON and H. E. HUXLEY, 1955) and I shall therefore give only a short account of it here. This forms the first section of the article, while the second reviews older observations and opinions in the light of this work. The third section deals with activation and the function of the *Z* membrane; in the fourth, there is an account of the hypothesis to which I have just referred, and in the fifth are discussed various phenomena for which fresh interpretations are suggested by the structural data.

The article makes no attempt at completeness as a review, and it contains a higher proportion of historical matter and of speculation than is customary in review articles. It deals mostly with structure and with the physical changes that accompany contraction, while the chemical and electrical events are referred to only in so far as they have a direct bearing on these central topics.

I. Structure of the Myofibril

I. 1. *Interpretation of the striations*

The essential facts about the structure of a striated muscle fibre at rest that were established by the nineteenth-century microscopists are the following.

(1) The transversely striated appearance of a fibre examined in ordinary light is due to an alternation of zones of higher and lower refractive index.

(2) The interior of the fibre consists of fibrils embedded in a varying amount of probably liquid sarcoplasm, which also contains nuclei and granules. When the fibrils are separated from one another, each shows the banding pattern characteristic of the whole fibre.

(3) The zones of higher refractive index (*Q* or *A* bands) are birefringent (uniaxial, with the optic axis parallel to the length of the fibre, and with positive sign), while the zones of lower refractive index (*J* or *I* bands) are optically isotropic or nearly so.

(4) Each *I* band is bisected by a narrow line of high refractive index, which appears to represent a more or less continuous membrane stretching across the fibre and attached to the sarcolemma.

(5) The centre of the *A* band sometimes has a lower refractive index than the edges, and is then called the *H* band.

These points were first clearly stated by Bowman (1840) for (1) and (2), Brücke (1858) for (3), Dobie (1849) and Krause (1869) for (4), and Hensen (1869) and Engelmann (1873a) for (5). They were clearly understood by Engelmann, Rollett, and others who investigated fresh fibres by visual microscopy towards the end of the last century, but the first has been a source of much confusion in more recent times. The striations do not differ appreciably in the absorption of light; consequently they are invisible if the fibre is examined in ordinary light with a wide condenser aperture. The refractive index difference between *A* and *I* can be made to show up by stopping down the condenser: the striations then become visible but the appearance depends on the focusing of the microscope. If it is focused exactly on a thin part of a fibre, almost nothing is seen; if the microscope tube is raised slightly, high refractive index regions appear bright, but if the tube is lowered the image is reversed and high refractive index regions become dark. To speak of the "dark" and "light" bands is therefore meaningless unless the position of focus of the microscope is specified; in the last century it became customary to use the low position ("tiefe Einstellung"), so that *A* and *Z* could be called dark; it is convenient but also potentially misleading that this agrees with the appearance of a fibre stained with the commonly used basic dyes. The interpretation of the image of a "phase object" such as this, in which light is retarded but not absorbed,

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became less important with the improvement of section-cutting and staining methods, and it seems nowadays not to be universally understood even by those who work with the microscope. The interpretation is still more difficult in a thick specimen such as a frog muscle fibre, and it is very difficult to tell, for example, whether the dark bands seen by BUCHTHAL *et al.* (1936) were in fact the *A* bands as these authors assumed.

I. 2. *Structure of the A, I and H bands*

It was widely supposed before the second war that the high refractive index and birefringence of the *A* bands were due to the presence there in a more or less solid state of the "myosin" which can be extracted by strong salt solutions or dilute acids (KÜHNE, 1864; DANILEWSKY, 1881). Although this view was strongly supported by the quantitative work of NOLL and WEBER (1934), it seems to have been discarded after the war, partly because the "myosin" had by then been shown not to be a single substance, but chiefly, I think, because it was difficult to reconcile this localization of what was thought to be the contractile

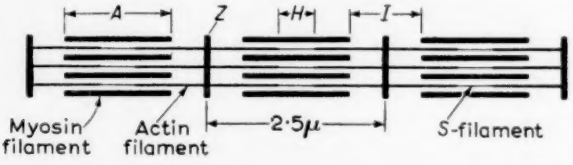

Fig. 1. Diagram showing the arrangement of the filaments, within a myofibril, which has been suggested by recent observations described in the text. The degree of stretch corresponds to the extended length in the body. Transverse dimensions are enormously exaggerated in comparison with longitudinal ones. When the length of the muscle is changed, the actin and myosin filaments slide past one another in each of the zones where they overlap, and only the *S*-filaments are actually stretched or shortened.

substance with the current idea that contraction was produced by the folding of protein chains that extended throughout the length of the muscle. Recently, good evidence has appeared that these characteristics of the *A* band are indeed due largely to the localization there of the myosin (as opposed to actin) component of the old "myosin." At the same time, observations on the changes of the striations during stretch and contraction, and electron microscope studies of thin sections of muscle, led to ideas on the structure of the myofibril that fit in well with a localization of the chief materials of the fibril in different parts of the system of striations.

The resulting picture of the structure of a fibril is shown schematically in Fig. 1. The main features of this were arrived at independently by

H. E. HUXLEY and HANSON (1954) and by A. F. HUXLEY and NIEDERGERKE (1954), although more of its details are due to the former than to the latter authors. Fig. 1 is in fact almost the same as the corresponding diagram of HANSON and H. E. HUXLEY (1955). Most of the evidence on which this scheme is based has been thoroughly reviewed by HANSON and H. E. HUXLEY (1955); I shall therefore do no more than summarize it here.

The essentials of the scheme shown in Fig. 1 are:

(1) The *A* band owes its high refractive index and its birefringence to a set of rods or filaments arranged alongside one another. The length of each of the rods is constant at about 1·5 μ so long as the muscle does not shorten enough to bring the ends of the rods into contact with the *Z* lines.

(2) A second set of filaments extend from the *Z* line through the *I* and into the end of *A*, as far as the beginning of the less dense *H* region. These keep a constant length of about 1·0 μ so long as the muscle does not shorten to a sarcomere length below 2 μ.

(3) When the muscle lengthens or shortens, the filaments of these two types slide past one another.

(4) When the fibre shortens so much that the *I* filaments of the two ends of one sarcomere come into contact, they shorten further by folding or crumpling near the point where they have come into contact, and possibly at other places. Similarly, the *A* rods may fold at their ends if the fibre shortens so much that they come into contact with the *Z* line.

(5) Myosin is a principal constituent of the *A* rodlets, and actin of the *I* filaments.

(6) Corresponding *I* filaments of the two ends of a sarcomere are joined by a very extensible connection, the *S* filament.

(7) In transverse section, the filaments are arranged as shown in Fig. 2.

The chief points of the evidence which led to this picture being put forward are as follows:

(1) *Existence of two sets of filaments.* The two sets of filaments are beautifully shown in the region of the *A* band where they overlap, in electron micrographs of both longitudinal and transverse sections of frog and rabbit muscle (H. E. HUXLEY, 1953a).

(2) *Constancy of length of A-band rodlets.* (*a*) A. F. HUXLEY and NIEDERGERKE (1954) showed that, in a living isolated muscle fibre from the frog, photographed with an interference microscope, the *A* band stays of constant width, within the errors of measurement, during stretch, passive shortening and quick and slow contractions, so long as the sarcomere length does not fall below 1·8–2 μ. They also found the same in passive stretch and relaxation using polarized light (unpublished; see p. 266).

(*b*) H. E. Huxley and Hanson (1954) similarly found by phase contrast microscopy that the width of the *A* bands of fibrils from glycerinated muscle is independent of sarcomere length, in stretch and in contraction induced by ATP treatment, so long as the sarcomere length did not fall below 1·7 μ.

(*c*) Harman (1954) recorded spontaneous contraction and relaxation of isolated fibrils by cine photography with the phase microscope, and

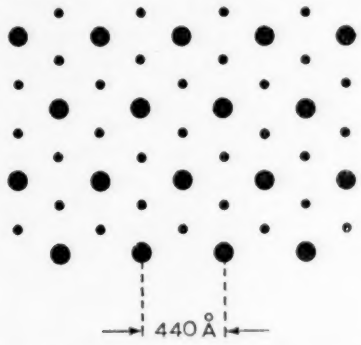

Fig. 2. Diagram showing the arrangement of the filaments in a cross-section through the outer part of an *A* band, where both myosin (larger) and actin (smaller) filaments are present. The dimensions given are appropriate for a muscle at its extended length in the body.

found that shortening took place by approximation of the *A* bands, with obliteration of the *I* bands.

(3) *Constancy of length of I filaments.* The gap between the *I* filaments belonging to the two ends of any one sarcomere shows up as the *H* band in the intact fibril, and as an almost completely empty region when the *A* filaments have been dissolved away. In both cases the gap increases in width as the fibre is stretched (H. E. Huxley and Hanson, 1954), and the length of the filaments that remain after extraction of the *A* filaments is independent of the degree of stretch (Hanson and H. E. Huxley, 1955, p. 247). In living muscles under the interference microscope, the *H* zone is conspicuous only when the fibre is moderately stretched (neither shortened not greatly stretched), as would be expected on this picture, but the optical conditions are not good enough to measure its width.

(4) *Constancy of X-ray spacings.* The 415 Å period of the low-angle X-ray diagram was found by H. E. Huxley (1952, 1953b) to be unchanged when the muscle was stretched. A spacing of about this value is seen in both *A* and *I* bands under the electron microscope (Hall, Jakus and Schmitt, 1946; Draper and Hodge, 1949), so it is

not clear whether the X-ray observation is really evidence for constancy of length in the *A* or the *I* filaments, or both. The high-angle X-ray pattern also appears to be little affected by stretch or contraction (ASTBURY, 1947).

(5) *Location of myosin in A, and actin in I, filaments.* HASSELBACH (1953) and HANSON and H. E. HUXLEY (1953) found that solutions known to dissolve myosin would readily remove most of the *A* band from fibrils of rabbit muscle; the central part of the *A* band disappeared only on prolonged extraction. HASSELBACH showed by viscosimetry that the material that went into solution was about 95 per cent myosin and 5 per cent actin. The *I* regions of the fibrils are not much affected by this extraction, and the *A* region is reduced to the same density as the *I* (except that, if the fibril is sufficiently extended to possess *H* bands, these become almost empty after the extraction). The fact that the actin is known to be present in untreated fibrils makes it probable that the remaining filaments consisted largely of actin; this was confirmed by HANSON and H. E. HUXLEY (1955), who showed that they disappeared on treatment with a solution known to dissolve actin.

(6) *Behaviour of filaments at short sarcomere lengths.* At sarcomere lengths between about 1·6 and 2·0 μ, the *A* band retains its ordinary length of 1·5 μ, but the *I* filaments are too long to do this. They appear to shorten by localized folding and not by uniform contraction, since narrow dense bands appear, particularly at the centre of the *A* band where the ends of adjacent *I* filaments meet (H. E. HUXLEY and HANSON, 1954; A. F. HUXLEY and NIEDERGERKE, 1954). On still further shortening, the dense bands at the *Z* lines become exaggerated as "contraction bands," which can naturally be interpreted as due to folding of the ends of the *A* filaments where they meet the *Z* line proper.

(7) *Existence of S filaments.* H. E. HUXLEY and HANSON (1954; HANSON and H. E. HUXLEY, 1955) found that a fibril from which the *A*-band material had all been dissolved away could still be stretched, leaving an apparently empty gap at the middle of each sarcomere, and would shorten again passively when released. There must therefore be some highly extensible connections between the *I* filaments of the two ends of any one sarcomere.

(8) *Arrangement of the filaments.* The arrangement shown in Fig. 2 is taken from H. E. HUXLEY's (1953a) very beautiful electron micrographs of transverse sections of rabbit and frog muscle. To a large extent, this arrangement was suggested by the same author's low-angle X-ray observations (1952, 1953b), which are also the source for the dimensions.

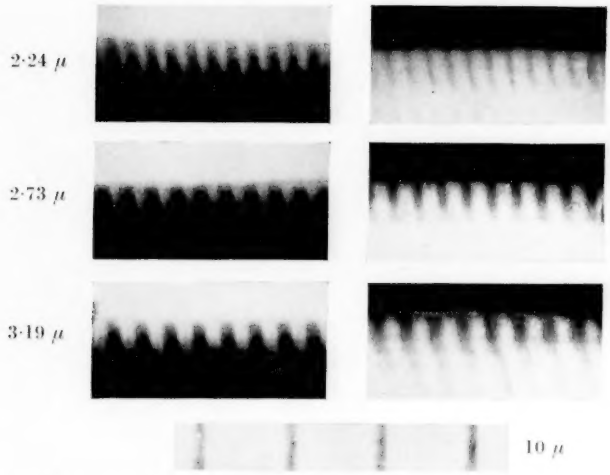

Fig. 3. Polarized light photomicrographs of the edge of an isolated living muscle fibre from the frog, unstimulated. *Right*: no compensation: background and *I* bands dark, *A* bands bright. *Left*: compensated so as to make *A* bands appear dark; background and *I* bands bright. Three degrees of stretch are shown, the sarcomere length in each case being indicated to the left of the photographs. The width of the *A* bands is seen to be unchanged while the *I* bands increase in width as the fibre is stretched. Water immersion objective, n.a. 0·75; condenser aperture limited by a square whose side (parallel to the axis of the fibre) gave an n.a. of 0·41. From a cine film taken in collaboration with Dr. R. E. Taylor.

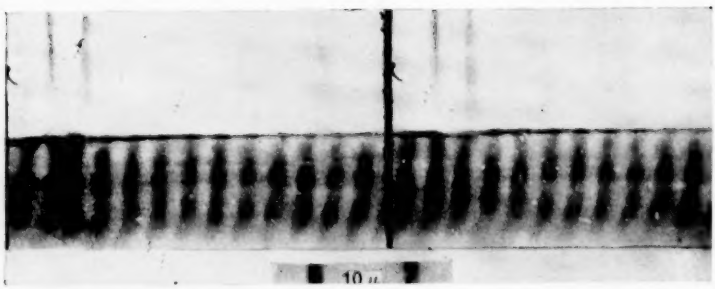

Fig. 4. Shortening of a single *I* band induced by depolarizing a small area of the membrane with a micropipette (tip diameter 4 μ) in contact with the fibre surface (the tip of the pipette is not well shown in this reproduction). Two successive frames from a cine film, 16 frames/sec; *right*, just before, and *left*, just after, the start of a negative pulse applied to the pipette. Isolated fibre from the frog; polarized light, compensated so that *A* bands appear dark; other optical conditions as in Fig. 3. Reproduced by permission of the *Journal of Physiology* from A. F. Huxley and Taylor, 1955b.

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I. 3. *Discussion of the evidence*

This evidence appears sufficient to show that the scheme under discussion is correct at least in its main outlines. There are obvious deficiencies, however: for instance, isolated fibrils contain tropomyosin and at least one unknown protein as well as actin and myosin (PERRY, 1953), and the material at the centre of the *A* filaments evidently differs in some way from that composing the ends which are easily removed by fluids which dissolve myosin. Points of this kind do not however alter the main thesis that changes of length involve two sets of filaments, one composed largely of myosin and the other largely of actin, sliding past one another.

Some of the important points in this evidence have however been recently questioned. HODGE (1955) has published some extremely clear electron micrographs of dipteran flight muscle in cross-section which show only a single set of filaments in the *A* bands. He regards this as contradicting H. E. HUXLEY's (1953a) evidence for a double set, but it seems unsafe to deny the existence of a structure on the grounds that it has not been seen in a particular preparation treated in a particular way. The material and the treatment are both very different from H. E. HUXLEY's, and it would be most valuable to discover what is the reason for the difference between their electron microscope pictures. A clue may perhaps be given by the fact that in his X-ray studies H. E. HUXLEY found evidence of the secondary filaments only in muscle that was in rigor, not in fresh muscle. HODGE also claims that his longitudinal sections show that the filaments of the *A* and *I* bands are continuous with one another. Apart from the fact that his *I* bands are not well preserved and the continuity is not easy to trace, at any rate in the published reproductions of his micrographs, this is again negative evidence in that filaments originally separate might become adherent during the preparation. It is clear however that these points need further investigation.

Another criticism of the evidence presented here concerns the interference microscope observations of A. F. HUXLEY and NIEDERGERKE (1954). BUCHTHAL *et al.* (1936) found, on isolated frog fibres under the ordinary light microscope, that the *A* band appeared to be more extensible than the *I*, which is the reverse of the findings reviewed above. As mentioned on p. 261, measurements of this kind on a thick specimen like a frog fibre (approx. 100 μ diameter) in ordinary light are not reliable; this was the reason for developing the interference microscope used by A. F. HUXLEY and NIEDERGERKE. But CARLSEN and KNAPPEIS (1955) now claim to have substantiated this result using polarized light, with which it is certainly possible to obtain a much more trustworthy image than with ordinary light. They agree that in fixed

or glycerol-extracted material the *A* band appears to stay of constant width, but state that in fresh isolated fibres from the frog the birefringent regions increase in width during stretch more than the isotropic bands. If this was true, it would invalidate much of the evidence I have quoted. But NIEDERGERKE and I also used polarized light on living fibres on several occasions, and found the *A* band width to be independent of the degree of stretch, just as we did with the interference microscope; Dr. R. E. TAYLOR and I have confirmed this again since the appearance of CARLSEN and KNAPPEIS's paper. A series of photomicrographs showing the constancy of width of the birefringent band is shown in Fig. 3. There is therefore a direct disagreement between CARLSEN and KNAPPEIS's results and our own; it is impossible to say what is the cause of the discrepancy, since CARLSEN and KNAPPEIS do not state the conditions under which their observations were made (numerical aperture of objective and of illuminating cone; whether compensation was used or not; thickness of specimen) and do not publish any of their photographs. It is possible that they used too low a condenser aperture; I have found with frog fibres of ordinary thickness that polarized light may then give an image almost indistinguishable from the ordinary light image. NIEDERGERKE and I did not publish any of our polarized light results in 1954 because this method, though better than methods using ordinary light, is less reliable than the interference method for thick specimens.*

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II. EARLIER EVIDENCE ON THE WIDTH OF THE *A* BAND AND ITS COMPOSITION

Some of the evidence for the idea that filaments of actin and myosin slide past one another when a muscle changes length was given in the last section. This included only the recent evidence which actually led to the idea being put forward. Several of the separate points that are established by this evidence (e.g. constancy of *A* band width, presence of myosin in *A* bands) were repeatedly suggested, and often clearly stated, both in the voluminous literature of last century and in more recent work. Probably none of this old evidence is important at the present day for helping to decide how far this idea is correct, but it is perhaps instructive to examine some of this work in the hope of seeing how it came about, in the first place, that the idea we are

* The reason is that the oblique rays in the plane of the long axis of the fibre traverse a greater thickness of muscle substance than the oblique rays in the plane perpendicular to the fibre; the total retardation of the extraordinary component by the parts of the fibre which are above and below the plane of focus is therefore greater for the former than for the latter groups of rays. The corresponding error with the interference microscope is eliminated by adding protein to the immersion fluid so as to bring its refractive index close to the average value for the fibre contents; there is no equivalent procedure for the polarizing microscope.

discussing was not put forward much sooner, and in the second place, that even the separate points which had formerly been accepted, and which are now confirmed, were generally rejected or disregarded from the end of the second war, or in some cases earlier, until a year or two ago. Many of the old observations are also of considerable interest in themselves, and have not been repeated in recent years.

II. 1. *Constancy of A band width*

It seems to have been generally agreed throughout the second half of last century that the width of the *I* band varied more than that of the *A* when fibres or fibrils in different degrees of stretch or contraction were compared. Some of the sources of this opinion are the following:

(1) Dobie (1849) reported that the band with lower refractive index (*I*) was extremely narrow in fibrils which were slightly shortened, and could sometimes be lengthened by stretching the fibril if the preparation was very fresh.

(2) Krause (1869, p. 172) observed that the width of the *A* band of vertebrate muscle fibres, which he gave correctly as 1·5 μ (p. 12), did not decrease during contraction.

(3) Engelmann (1880) measured the width of the birefringent bands (*A*) of fibres of fixed insect muscle. In "contraction waves" he found that *A* changes less than *I* on shortening; there was very little change in *A* during shortening to 60 per cent of the initial length; beyond that, *A* and *I* both shortened but *I* continued to form a reducing proportion of the muscle length.

(4) Kölliker (1888, p. 706) states that *A* enlarges at the expense of *I* during contraction, until neighbouring *A* bands come into contact with the intervening *Z*.

(5) Retzius (1890, p. 72) observed, in fixed and stained preparations of muscle from beetles, that contraction (shortening) is associated with first a narrowing, and then complete disappearance, of the *I* band (defined as the weakly stained region) before contraction bands appear.

(6) Rollett (1891) states that the low refractive index band becomes relatively longer in passive stretch (p. 61) and shorter on contraction (p. 69).

(7) Heidenhain (1911, p. 623) states that the *I* band is more extensible than *A*.

The testimony on this point is thus remarkably uniform. With the exception of Krause, however, none of these authors laid particular stress on this phenomenon; more emphasis was generally placed on the changes which occur in more extreme degrees of contraction (formation of contraction bands, etc.). Krause, however, on the basis of the apparent constancy of the width of the *A* band, put forward the theory

that the high refractive index and the birefringence of the *A* band were due to the presence there of submicroscopic rods in a parallel arrangement. The length of these rods was supposed to stay constant (1·5 μ) as the muscle shortened, their lateral separation increasing so as to keep the volume of the whole fibril constant. KRAUSE believed that the *I* bands were liquid during life, so that this fluid was supposed to enter the spaces between the *A* rodlets as they moved away from one another during contraction. He did not specify the forces that produced these movements, but suggested that the rods of each *A* band attracted those of the neighbouring *A*'s.

In recent years, however, it has been generally believed that contraction takes place in the *A* band (e.g. FULTON, 1955, p. 129). How then has a universal and, it now appears, well-founded opinion been reversed, in spite of having been embodied in a theory of contraction by one of the most distinguished of the nineteenth-century microscopists?

The most important factors in this change seem to have been two theoretical points raised by ENGELMANN. In the first place, he dismissed KRAUSE's theory on the ground that muscles can shorten far beyond the point at which the *A* bands of adjacent sarcomeres would come into contact if they stayed of constant width (ENGELMANN, 1873b, p. 161). This is admittedly a difficulty, though it should have been clear, independently of any theory, that the shortening process changes in some way at the stage where contraction bands begin to form. The second theoretical point is that ENGELMANN enunciated the principle that all formed contractile elements are birefringent (see for example ENGELMANN (1906), which is largely a review of his own work on muscle). From this generalization, which is probably valid as it stands, and is clearly of great significance, he concluded that the birefringent parts of a striated fibre must be the contractile parts, and hence that they must be the parts which shorten during contraction. He thus seems to have persuaded himself of the reverse of what he had found by direct measurement; in his article of 1906 he refers only in the most cursory way to his paper of 1880. It is worth noting two features of this argument: first, that an experimental observation has been overthrown on theoretical grounds, and second, that the argument involves the tacit assumption that muscle contains a single "contractile substance" which shortens during contraction. Both of these points will turn up again in a different connection (p. 271).

It is these opinions of ENGELMANN's that seem to have been generally accepted, but it is difficult to say how far this was due to his authority and how far to the well-known paper that was published by HÜRTHLE in 1909. He obtained cine-photographs of spontaneous contractions in insect muscle fibres, mostly with polarized light, and claimed that his results both confirmed ENGELMANN's claim that the shortening

occurs in the *A* band, and contradicted the generally accepted view that a "reversal of striations" (development of dense bands in the regions where *I*'s had been in the resting fibre) takes place when muscles shorten greatly. But the difference between his and his predecessors' results seems in fact to lie in the condition of his fibres in the resting state. His *I* bands appear to have been about $\frac{1}{8}$ the width of his *A* bands, while the usual figures given for this ratio are $\frac{1}{2}$ or more. The only explanation that suggests itself for this difference is that his fibres, even in the resting state, were already shortened almost to the point where contraction bands are formed, and further shortening must necessarily involve the *A* band. Similarly, he did not find a reversal because in these fibres at rest the *Z* line (with the accompanying *N* bands, which are composed of interstitial granules) appeared as the densest part of the sarcomere, this being no doubt connected with the narrowness of *I*. He observed the formation of dense contraction bands at the level of *Z* (in agreement with all other observers) but did not regard this as constituting a reversal because they were at the same position as the densest part of the resting pattern. His differences from former observations are therefore more apparent than real, and depend on some imperfectly explained difference in the initial state of the fibres; nevertheless, his conclusions have been widely quoted not only as supporting ENGELMANN's contention that shortening takes place only in the *A* bands (e.g. MEYERHOF, 1930, p. 295) but as disproving the reality of the reversal phenomenon and thence of the formation of contraction bands.

The other well-known work which appears to support ENGELMANN's view is that of BUCHTHAL, KNAPPEIS and LINDHARD (1936). They photographed isolated fibres from the frog in ordinary light, and found that their dark bands changed in width more than their light bands, during both passive stretch and isotonic contraction. As was mentioned on pp. 261 and 265, this is an extremely difficult specimen from the optical point of view, and I can only conclude, from the disagreement with our interference microscope results (A. F. HUXLEY and NIEDERGERKE, 1954) that they were not justified in assuming that their dark band corresponded accurately in width to the actual high refractive index bands of the fibres. This work was, however, generally accepted as further evidence in support of the view that it is the *A* band which is contractile. The observations of HORVATH (1952), which appear to show that the *A* and *I* bands of a glycerol-extracted fibre maintain a constant ratio during contraction, are subject to the same criticisms.

With the advent of the electron microscope, these confusions ought to have been cleared up at once, but they were not. HALL, JAKUS and SCHMITT (1946, p. 38), in their well-known paper, do indeed state that the *A* band hardly changed in width on passive stretch, but they also

state that this confirms BUCHTHAL *et al.*'s results, while in fact it is in direct contradiction with them. Unlike the other features of their photographs, this particular result did not attract much attention; even the authors themselves do not mention it in a subsequent review article (SCHMITT *et al.*, 1947). DRAPER and HODGE (1949) published very good micrographs of separated fibrils from fixed toad muscle; measurement of their plates shows that the *A* band was very constant at about 1·5 μ width in all fibrils that were not so much shortened as to produce contraction bands, but the authors do not comment on this. HOFFMANN-BERLING and KAUSCHE (1950), using similar preparations from frog muscle, did clearly state that only the *I* band extended noticeably on stretching, though all their absolute dimensions are much too small ($1\frac{1}{2}$—2 times), suggesting that either their preparations had shrunk or the magnification was not correctly determined. BLUHM and SITARAMAYYA (1951) also found the width of the *A* band in fibrils from rat diaphragm to be unchanged on stretch and during isometric contraction, but to shorten somewhat during isotonic contraction. These two papers do not appear to have received the notice they deserved. The figures given by PHILPOTT and SZENT-GYÖRGYI (1953) for the ratios of widths of the various bands in thin sections do show greater changes in *I* than in *A*, but *A* does not seem to stay constant. These results are again unsatisfactory, because the authors assumed without a check that the absolute sarcomere length in each photograph was given by the degree of stretch applied to the muscle before fixation.

It is thus unfortunately the case that there is at present no really satisfactory electron-microscope evidence on the question whether the *A* band width remains constant; on the whole, this idea is supported by the electron-microscope work, and is certainly not excluded by it. DEMPSEY *et al.* (1946), in their histochemical study of muscle, also found that the *I* band became narrower in moderate contraction, until it almost disappeared.

II. 2. *Localization of myosin*

"Myosin" was first extracted from muscle by KÜHNE (1864), who named it and characterized it by its solubility properties and by the great ease with which it is denatured. ENGELMANN (1873b, p. 174) mentions in passing that he believes myosin to be the anisotropic substance, "on account of its histochemical reactions." T. H. HUXLEY (1880), in a general description of muscular tissue, was able to write: "In fibres which have been acted upon by solution of salt, or dilute acids, the inter-septal zones [*A* bands] have lost their polarizing property. As we know that the reagents in question dissolve the peculiar constituent of muscle, *myosin*, it is to be concluded that the inter-septal substance is chiefly composed of myosin." (The "septa" on which his

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nomenclature of the striations is based are the *Z* membranes; the "inter-septal zones" correspond to the *A* bands, and are contrasted with the non-birefringent "septal zones" which correspond to the *I* bands.) Similar observations were made by SCHIPILOFF and DANILEWSKY (1881), who showed also that the high refractive index of the *A* bands disappeared when the myosin was extracted. They also found that a drop of myosin solution became birefringent on drying.

This view seems to have been generally adopted (e.g. the review by BIEDERMANN, 1927, p. 427), but there seems to have been no fresh evidence until 1930 when VON MURALT and EDSALL showed that myosin solutions gave flow birefringence, and that the particles were of a length roughly equal to the width of an *A* band (EDSALL, 1942). Finally, in 1934 WEBER made oriented threads of myosin, and NOLL and WEBER showed that their birefringence would account for both the intrinsic and form components of that of muscle.

The interpretation of these results was of course complicated by the discovery in SZENT-GYÖRGYI's laboratory (STRAUB, 1943) of the composite nature of the material that had formerly been known as myosin, but there does not seem to be any justification for the way in which the evidence for localization of at least one component came to be disregarded. The chief reason seems to have been that the interactions between actomyosin and ATP provided plausible theories of contraction which required both the actin and the myosin to be uniformly distributed along the whole of the fibrils, so that, on activation, they could form actomyosin filaments which were supposed to shorten by folding under the influence of ATP. The only evidence adduced in favour of this uniform distribution was that the early electron micrographs, especially those of HALL *et al.* (1946), appeared to show continuous filaments running through both *A* and *I* bands. ROZSA, SZENT-GYÖRGYI and WYCKOFF (1950) did suggest that the filaments might be actin and that the extra material which causes the electron-scattering power of the *A* band might be myosin, but SZENT-GYÖRGYI (1953, p. x) later gave up this view.

The position appears to be closely parallel to that which led, after 1910 or so, to the assumption that shortening takes place in the *A* band (see p. 268). The experimental evidence, which on its own account requires at least one component of actomyosin to be localized in the *A* bands, was discarded on theoretical grounds, and the argument again involved the tacit assumption that there is a single contractile substance (actomyosin) which itself shortens during contraction. A further parallel is that in both cases the theoretical view received support from some technically very impressive observations (in the first case, HÜRTHLE's cine-photomicrographs; in the second, the early electron-microscope observations) which, however, would probably not have

been adequate to overthrow the older evidence without the theoretical backing.

II. 3. *The idea of sliding molecules*

The idea that passive stretch of a striated muscle takes place by long molecules sliding past one another, rather than by unfolding or stretching of chains, was put forward at least twice before the evidence discussed on pp. 262–264 had been obtained. FISCHER (1947, p. 787) made this suggestion on the ground that the birefringence of striated muscle changes very little on stretch, and MORALES (1948) did so in order to explain the great extensibility of resting muscle and the absence of change in the wide-angle X-ray pattern; all these appear to be important pieces of evidence. Neither of these authors assumed that active shortening took place by sliding. MORALES did point out that the decrease of active tension with stretch might be accounted for by the decrease of the zone of overlap, but his argument differs from that of A. F. HUXLEY and NIEDERGERKE (1954) in that he postulated only that the strength of connection would decrease, not the number of sites where tension is generated. BOZLER (1936) suggested that the behaviour of smooth muscle could best be explained by assuming that contraction involved changes in the relative position of the molecules, and not in their shape.

III. "ACTIVATION" AND THE *Z* MEMBRANE

III. 1. *The link between excitation and contraction*

A great deal is known about the electrical events which accompany excitation of a muscle fibre, and about the contraction process, but very little about the steps that link them together. The interior of a resting muscle fibre is electrically negative to the surrounding fluid by 60–100 mV, the potential difference existing across a very thin surface membrane. When the fibre is excited through its nerve supply, this potential difference undergoes a transient reduction or reversal. In the most familiar kinds of muscle fibre (those which are capable of an all-or-none twitch in response to direct electrical stimulation), this decrease of membrane potential ("depolarization") propagates itself along the whole length of the fibre by a process which is essentially the same as the conduction of an impulse along a non-myelinated nerve fibre. In other cases (the slow fibres of frogs, and many arthropod muscles) the depolarization spreads passively for only a short distance from each neuromuscular junction, but the whole of the length of each muscle fibre is nevertheless brought into action because it receives numerous nerve terminations distributed along its length. In all cases, however, the electrical change is a reduction (or reversal) of the

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potential difference across the surface membrane. It is also found that, if this potential difference is reduced by any other treatment of the fibre (passage of current; KCl solutions; application of acetylcholine to an endplate), the part of the fibre which is depolarized contracts (KUFFLER, 1946). This suggests strongly that the membrane potential change is the essential thing for setting off the contractile process, but if so there is the difficulty of explaining how this electrical change, which can exert a direct influence only on the membrane itself, is able to cause fibrils in the interior of the fibre to contract. The distances involved are considerable: the fibres of skeletal muscle of the frog are commonly over 100 μ in diameter, while in Crustacea the fibres reach 0·5 mm or more. Further, A. V. HILL has shown (1949c) that the link cannot be a substance which is released at the surface membrane and reaches the fibrils by a simple diffusion process, since this would be too slow to account for the very rapid onset of heat production and of the contraction itself.

A suggestion which has occurred to many people is that it is not the membrane potential change, but the currents in the interior of the fibre, that are the immediate cause of "activation" of the contractile substance. If this were the case, there would be no difficulty in explaining the speed of the onset of contraction. But KUFFLER (1946) pointed out that contraction is produced even by a uniform depolarization of the fibre surface (e.g. by immersion in a solution with raised potassium concentration) when there should be no current flow; and STEN-KNUDSEN (1954) has shown that when a muscle is caused to contract by applied current in conditions where an action potential is not produced, the contraction is confined to the neighbourhood of the cathode, where the membrane potential is reduced, while the region midway between the electrodes, where the current density inside the fibres is a maximum, is passively stretched.

It is thus highly probable that the membrane potential change itself is the essential factor for causing activation of the contractile material, and we are left with the problem of the nature of the link between membrane and fibrils.

III. 2. *The Z line*

The *Z* line is a narrow band of high refractive index at the middle of the *I* band. It is widely, though not universally, believed to represent a more or less continuous membrane which runs across the whole fibre, uniting the fibrils to one another and to the sarcolemma. Many authors have ascribed to it a purely mechanical function, keeping the striations of the separate fibrils in register and perhaps transmitting tension in some way to the sarcolemma; the difficulties in this idea have been well stated by BENNETT (1955). But its apparent continuity

and attachment to the sarcolemma also suggested to TIEGS (1924; MATTHAEI and TIEGS, 1955) that its function might be connected with the activation of the contractile fibrils. In TIEGS's view, the action potential itself travels along the *Z* membrane, which he believes to be invariably a spiral structure; it seems clear from electrophysiological work that this cannot be the case, at any rate in normal contractions of vertebrate muscle, where the action potential is clearly a self-propagating change in the potential difference across the surface membrane. However, the possibility remains that the *Z* membrane is concerned with conveying to the myofibrils the influence of a reduction of the potential difference across the surface membrane. BENNETT (1955, p. 54), on the basis of electron microscope observations, has recently put forward the theory that an excitatory influence is conducted inwards from the sarcolemma by the sarcoplasmic reticulum and conveyed to the myofibrils at the *Z* lines (see also p. 276 below).

Dr. R. E. TAYLOR and I recently devised an experiment to test the hypothesis that the *Z* membrane is specifically concerned in the inward spread of activation (A. F. HUXLEY and TAYLOR, 1955a,b). If this is the case, then presumably it is the potential change at the attachments of *Z* membranes to the sarcolemma that counts, and no contraction would be expected to follow a potential change which involved a small area of membrane lying entirely between two adjacent *Z* line attachments. We achieved this situation by bringing a pipette with a squared-off tip, diameter about 2 μ, into contact with the surface of an isolated muscle fibre that could be observed and photographed through a polarizing microscope. The potential difference across the small area of membrane opposite the tip of the pipette could now be reduced by making the interior of the pipette electrically negative relative to the bathing fluid. The resistances at the tip of the pipette, both that of the "seal" of the tip to the fibre and that of the small area of membrane itself, are high enough for the current entering or leaving the pipette to be too small to produce appreciable changes in potential either inside the fibre or in the bathing fluid outside the pipette; this is true even if the membrane opposite the tip were to undergo an action potential.

The muscle fibre was slightly stretched, so that the *Z* lines were about 3 μ apart; the 2 μ pipette could therefore be placed either opposite a *Z* line (centre of an *I* band) or in the space between two adjacent *Z*'s (opposite an *A* band). In the former case, we found that a moderate depolarization (20–40 mV) caused a localized contraction, but in the latter case, a potential several times larger could be applied with no result at all. Further, when shortening occurred, it was confined to the *I* band opposite the pipette, and spread only a moderate distance inwards from the surface of the fibre. A contraction of this

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kind is shown in Fig. 4; the pipette in this case was 4 μ in diameter, which is about the largest that can be used without overlapping more than one *Z* line, though it is too large for the space between two adjacent *Z*'s. The contraction spreads further inwards than was the case with the 2 μ pipette, but is otherwise just the same. This photograph also illustrates in passing the fact that the shortening takes place exclusively in the *I* band, the two dark *A* bands being drawn together without changing in width.

These results are entirely consistent with the hypothesis that we started from, namely that the influence of membrane depolarization which activates the contractile substance is conveyed along *Z* membranes. The experiment also shows that the resulting activation affects only the half-sarcomere on either side of the *Z* membrane in question; in Fig. 4 the contraction spreads about 10 μ inwards but does not affect the adjacent *I* bands which are only about 2 μ away. It is not actually proved that the visible *Z* membrane is the structure concerned; it may be that the activation is conveyed along some other structure near the middle of each *I* band. The anatomical evidence reviewed in the next section does however show that the *Z* membrane has the required characteristics, and it seems most probable that it is the structure concerned.

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III. 3. *Continuity of the Z membrane*

The *Z* line, which is seen as a narrow band of high refractive index in a fresh muscle fibre, is also very conspicuous in isolated fibrils, whether examined by phase microscopy or in the electron microscope. It usually appears as the densest part of the fibril, as regards refractive index in the light microscope, electron scattering in unstained and unshadowed electron-microscope preparations, and thickness in shadowed fibrils under the electron microscope. There is a good deal of evidence that these dense lines in the separate fibrils are connected to one another across the muscle fibre. This evidence falls into two groups; the first is from observations on fresh muscle while the second is from examination of fixed and stained preparations.

Several very convincing pieces of evidence in the first of these classes date from the last century. KRAUSE (1869, p. 11), SCHIPILOFF and DANILEWSKY (1881) and KÖLLIKER (1888) all observed that continuous structures remained at the position of the *Z* lines when the rest of the fibrils was removed by dilute acid; this is particularly notable in the case of KÖLLIKER's observations since he was using insect fibrillar muscle, in which the fibrils, or sarcostyles, are separated by large amounts of sarcoplasm rich in granules.

In the second class are the observations of ENDERLEIN (1899) and HEIDENHAIN (1911, p. 613), who were able to stain structures uniting

the *Z* lines of adjacent fibrils even where they were separated by a broad band of sarcoplasm, and also running from the sarcolemma to the *Z* of the most superficial fibrils. This was confirmed by VON BOGA (1937), who pointed out that the fine membranes uniting the *Z*'s of adjacent fibrils stain differently from the *Z*'s in the fibrils themselves. TIEGS (1955) has also demonstrated the continuity of *Z* in both fibrillar and non-fibrillar muscles of insects.

A somewhat similar conclusion is reached by BENNETT and PORTER (1953) and BENNETT (1955) from very beautiful thin sections of muscle under the electron microscope. They show connections between the *Z*'s of adjacent fibrils and between the sarcolemma and the *Z* lines of nearby fibrils, but these connections are very much thinner than the *Z* lines within the fibrils, and are easily distorted if the fibrils are displaced longitudinally in preparing the specimens. They regard these connections as part of a "sarcoplasmic reticulum" which exists in the spaces between the fibrils, and connects with the sarcosomes; this reticulum was well described by KÖLLIKER (1888) and RETZIUS (1890) in fibres stained by procedures in which the fibrils were dissolved out or were swollen until they did not interfere with the image of the sarcoplasmic structures.

It seems legitimate to conclude that there exists in the middle of each *I* band a structure which is connected to the sarcolemma, which runs right across the fibre, and of which the corresponding *Z* lines of the individual fibrils form a part. This whole structure may perhaps be called KRAUSE's membrane, or *Z* membrane, in contradistinction to the *Z* lines in an individual fibril (VON BOGA, 1937).

The existence of a "continuous" structure in this sense must not be taken to mean that there is a complete membrane, like a cell membrane, crossing the fibre in each *I* band. The electrical evidence clearly shows that such a thing does not exist, the fibre interior having a fairly low resistance to longitudinal current (KATZ, 1948). If the *Z* membrane is of the same nature as a cell membrane, having a low permeability for ions and a high electrical resistance, it must have extensive perforations through which ions can move; if it is continuous in the sense of not being perforated, it must offer only a very slight resistance to the movement of ions.

III. 4. *Mechanism of conduction along Z membranes*

If we allow ourselves to conclude from this evidence that each *Z* membrane is concerned with conveying an influence of surface depolarization to activate the half-sacromeres which lie on either side of it, the question at once arises, what is the nature of this influence, and how is it conducted. The only clue to this that the experiments provide is the fact that the contraction does not propagate itself inwards, but

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spreads for a distance which increases with the voltage applied and with the size of the pipette. The experiment does not prove conclusively that a self-maintaining inward propagation does not occur in a perfectly undamaged fibre, since the fibres had ceased to give ordinary all-or-nothing twitches by the time we managed to observe these local effects, and it is conceivable that there is normally a propagating mechanism which had by then been lost. But fibres in this state can give apparently normal twitches when the membrane is depolarized by applied current (BROWN and SICHEL, 1936; confirmed in unpublished work by Dr. R. E. TAYLOR and myself). There is therefore no need to postulate a self-propagating mechanism, and in any case our experiments show that a graded activation can spread quite a long way in. This seems to exclude such mechanisms as crystallization (HILL, 1949c) and "activated diffusion" (diffusion of a substance whose presence causes more of the same substance to be liberated) which have been suggested as mechanisms for the rapid activation of the fibre interior in response to surface-membrane potential changes, since they would be expected to be self-propagating. At the same time, the fact that the contraction did not reach nearly to the centre of the fibre would be expected on any mechanism that was not regenerative, since the pipette covered only a very small sector of the edge of the *Z* membrane, and the influence of depolarization on this small region was spread out over a broad front as it was conducted inward.

There is nothing at present to contradict the very tentative suggestion that the mechanism may be an electrical one. If the *Z* membrane contained channels whose lumen was connected to the external fluid, and whose walls had a high resistance, then the potential difference across the walls would follow that across the surface membrane of the fibre with a lag depending on the size of the channels, the capacity of their walls, etc. Rough calculations show that the lag would be very small even if the channels were as small as 100 Å diameter, and were insulated from the sarcoplasm by walls with a capacity of 1 μF/cm^2 such as is found in the membranes of many cells.

III. 5. *Longitudinal spread of activation*

There is also the question how a change spreading inwards along the *Z* membrane brings about the contractile process which presumably occurs in the nearest half-*A*-band on either side. The striking feature about this final stage of the spread of activation is that it goes no further than this: no shortening was seen in the *I* bands on either side of the one to which the pipette was applied. If diffusion of a substance from *Z* is responsible, then there must be something that limits the range of diffusion, e.g. the diffusing material might be used up in the first half of the adjacent *A* bands, or there might be an impermeable

layer at the position of *M*. The limitation of the spread of activation would however be directly explained if the change was some alteration in the actin filaments, which was conveyed along them from the *Z* line, since they terminate at or before the middle of the adjacent *A* bands. Other points which suggest that activation may be accompanied by a change in the actin filaments are mentioned on pp. 301 and 305.

III. 6. *The functional unit in striated muscle*

It is customary to take as the repeating unit of the striation pattern the sarcomere, which is defined as the region bounded by two adjacent *Z* lines. The experiments described here suggest that the functional units are centred on the *Z* lines, and not separated by them, so that the unit consists of a *Z* line with the *I* band in which it lies and the half *A* band on either side. We never saw a contraction involving one-half of an *I* band only (though our pipettes may not have been small enough to achieve this if it is possible in principle, and it is also conceivable that a contraction at one side only of the *Z* line might have been mistaken for a weak contraction involving both sides). Also, as mentioned above, we never saw any shortening of the neighbouring *I* bands such as would have occurred if contraction had taken place in both halves of each of the *A* bands adjacent to the point of stimulation. There are, however, occasional records in the literature of contraction waves in fixed preparations of insect muscle where the degree of contraction is strikingly different in the half-sarcomeres on either side of a single *Z* line (Rollett, 1891, fig. 10; Tiegs, 1955, fig. 39).

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III. 7. *Other functions of the Z line*

It was mentioned above that there are great difficulties (Bennett, 1955) in the idea that the *Z* membrane conveys the force of contraction to the sarcolemma, but it remains possible that the *Z* line has a mechanical function as well as being concerned in the activation of the fibrils. Within each myofibril there must be some structural link which keeps the myosin filaments alongside one another in each *A* band, and another link which keeps each set of actin filaments in register. The electron micrographs of Hodge (1955) show cross-links between the filaments of the primary array (presumably myosin) in the *A* band; it may be these that keep the myosin filaments aligned, but if so there cannot be corresponding cross-links between the actin filaments, or the sliding movement would be impossible: connections could only exist at the position of the *Z* line, where they would not interfere with the movement of the myosin filaments and their set of cross-links. It is therefore necessary to keep in mind the possibility that the *Z* line has also the function of keeping the actin filaments in alignment.

IV. A Hypothesis for the Mechanism of Contraction

Most, if not all, of the hypotheses that have been put forward in recent years as explanations of muscle contraction on the molecular level have assumed that protein chains, running longitudinally in the muscle, shorten by some kind of folding or coiling. These theories have differed in the type of folding assumed, and in the nature of the forces that were supposed to produce the folding, but have been remarkably uniform in assuming that shortening takes place in each filament at a number of points in series, i.e. the decrease in length of the whole filament is the sum of the amounts of shortening that have occurred at the different points of folding along its length, while the whole tension in the filament is exerted at each of these different links. This idea appears to have been supported especially by the changes of structure that were found by Astbury and Dickinson (1940) to occur when a myosin film is stretched or made to shorten, and by the appearance of continuous filaments which was suggested by the early electron-microscope studies of muscle. On the other hand it is very difficult to reconcile with the many lines of evidence, reviewed here and by Hanson and H. E. Huxley (1955), showing that the individual filaments do not change in length appreciably when a muscle contracts, anyhow over the greater part of its normal range.

If then we are to replace the hypothesis that shortening is generated by a filament folding at a number of places in succession, by the hypothesis that two types of filament slide past one another, how are we to suppose that this relative movement is brought about? So far, three broad suggestions have been made:

(1) The filaments move so as to increase the number of points at which some chemical interaction can take place between them (H. E. Huxley and Hanson, 1954).

(2) The filaments of one type are capable of small changes of length, but these take place cyclically, the attachments between the two filaments being broken and remade so that the overall result is relative movement between the filaments without appreciable shortening of either. This type of scheme, with the further suggestion that the cyclical changes might take place in the actin filaments by successive depolymerization and repolymerization, has been put forward by Hanson and H. E. Huxley (1955), and by Aubert (1955).

(3) A relative force between adjacent filaments of the two kinds is generated at each of a series of points within the zone in which they overlap (A. F. Huxley and Niedergerke, 1954; discussed also by Hanson and H. E. Huxley, 1955).

So far, there is nothing worthy of the name of evidence either for or against any of these three general possibilities. The second has the

advantage of bringing in some of the interesting properties of actin, while the third provides an explanation for the parallelism between the isometric tension generated in a tetanus and the width of the overlap zone, when a muscle is extended beyond its resting length (A. F. HUXLEY and NIEDERGERKE, 1954). The same authors pointed out that their scheme would account for the known correlation between narrowness of striation and speed of contraction, but this could probably also be fitted into either of the other schemes.

It is possible that high-resolution electron microscopy may help to decide between these alternatives, but it might not: some kind of structural link between the two sets of filaments is required on any of these schemes, and even if the links are seen, it may not be possible to say in which way they are acting. Another way of trying to decide between them is to follow out in detail the relationships between observable quantities (tension, heat production, etc.) that would be expected on each, and seeing whether any of the schemes can be excluded through failure to fit in with the experimental data. As I have stated them, the three types of mechanism are not specified in enough detail for making predictions which can be checked experimentally; before this can be done, a sequence of events linking the supply of chemical energy to the mechanical system must be specified, together with the factors which control the rates of the reactions. Schemes which are put forward in this amount of detail at the present time must necessarily be highly speculative, but may nevertheless be helpful both in showing how far it may be possible to go in explaining the known behaviour of muscle, and also—more important—in suggesting experiments which may exclude particular hypotheses or groups of hypotheses.

One such scheme (belonging to the third of the classes mentioned above) was worked out in some detail by the author in the summer of 1954. It fitted a number of the known properties of muscle fairly well, without many arbitrary postulates, and it therefore seems worth while to give an account of it here. It was framed primarily with reference to the "sliding" hypothesis and to the relations between load, shortening and heat production, but the chemical reactions provisionally assumed to underlie the steps of the contraction process have been chosen so as to be consistent with the main features of the behaviour of SZENT-GYÖRGYI's glycerinated muscle preparation (WEBER, 1955).

The hypothesis will first be stated in general terms, and then a mathematical treatment will be given in order to derive formulae for tension and heat production, as functions of the speed of shortening, for checking against the relationships found experimentally by A. V. HILL.

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IV. 1. *Statement of the hypothesis*

The general idea is that each of the filaments of one type (provisionally assumed to be the myosin filaments) has side-pieces which can slide along the main backbone of the filament, the extent of the movement being limited by an elastic connection. These sliding members can combine temporarily with sites on adjacent actin filaments, the connections being formed spontaneously but broken only by a reaction requiring energy to be supplied from metabolic sources. The essential feature of the system is that the rates of the reactions by which the

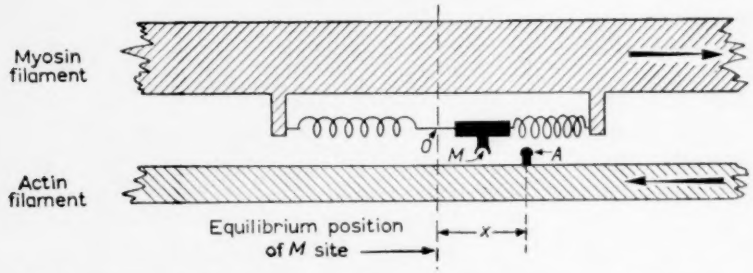

Fig. 5. Diagram illustrating the mechanism by which it is assumed that tension is generated. The part of a fibril which is shown is in the right-hand half of an A band, so that the actin filament is attached to a Z line which is out of the picture to the right. The arrows give the direction of the relative motion between the filaments when the muscle shortens.

connections are made or broken are assumed to depend on the position of the sliding member relative to the backbone of its myosin filament. This is not difficult to imagine if we suppose that the reactions are catalysed by enzymes which are fixed to the myosin filament, or perhaps actually form part of it.

The essentials of the scheme are illustrated in Fig. 5. The contractile element shown is to be thought of as lying in the right-hand half of an A band, so that the nearest Z line, to which the actin filament is attached, is off the picture to the right. During shortening, therefore, the actin filament moves to the left relative to the myosin filament. The distance of A, the active site on the actin filament, from O, the equilibrium position of the sliding element on the myosin filament, is denoted by x (positive if A is to the right of O). During steady shortening, x decreases at a constant rate. The rate constants for the reactions which make and undo the connection between A and M are denoted by f and g respectively; they depend on x in the manner shown in Fig. 6.

The system works as follows. Initially, the groups M and A are detached; M oscillates back and forth about its equilibrium position O as a result of thermal agitation. If A happens to be within the range

of positions where f is not zero (i.e. where the combination of M with A is catalysed), there is a chance that combination will take place; when this has happened the tension in the elastic element will be exerted on the actin thread. The region where this combination can occur is to the right of O, so that the tension in the elastic element is in the direction to help the muscle to shorten. The AM combination moves towards O as the muscle shortens; there is all the time a chance that

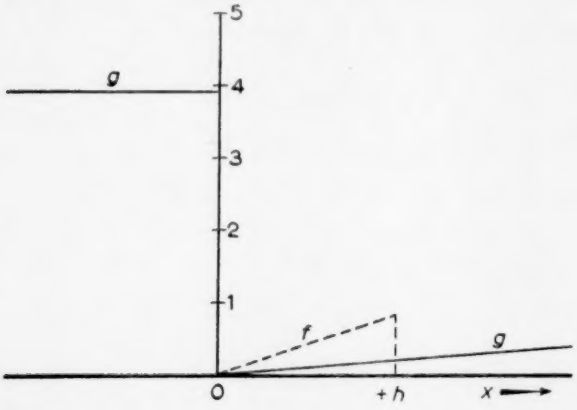

Fig. 6. Dependence of f and g on x. Dashes, f, i.e. rate constant for formation of links between actin and myosin by reaction (1). Continuous line, g, i.e. rate constant for breaking links by reaction (2). The unit of the ordinate scale is the value of $(f + g)$ at $x = h$.

the link will be broken, since g is finite everywhere (except at the point O), but the chance is small until the group passes O; g then increases greatly and the AM link is soon broken, preventing the tension in the elastic link, which is now in the reverse direction, from holding up the shortening of the muscle. At high rates of shortening, a large proportion of the links will not be broken in time to prevent a considerable resistance from being generated in this way; the speed of shortening of an unloaded muscle reaches its limit when this resistance just equals the force produced by the links to the right of O.

We have tacitly assumed that the combination of A and M took place spontaneously with the equilibrium in favour of the combined state; hence it is necessary to postulate that energy is provided in order to break the link. It would be in keeping with SZENT-GYÖRGYI's work and with recent work on glycerinated fibre models (WEBER, 1955) to suppose that this is brought about by some high-energy phosphate compound (denoted by XP) uniting with a site near A. After the A and M groups have separated, XP is split and its fragments are detached, restoring the initial conditions, except that now A is in a

position where f is zero and recombination is not catalysed and therefore does not occur. As the muscle shortens further, the A group we have been considering moves on to a region where it may combine with the next M group further to the left on the myosin chain, while the next A group to the right on the actin chain comes into the picture and is able to combine with the M group that is under discussion.

This sequence of events must be supposed to take place at a number of sites on each filament within each A band, the sites being staggered so that they come into action asynchronously as the muscle shortens. The overall tension in the muscle will therefore not fluctuate appreciably as the links are made and broken.

The reactions that have taken place during the cycle are:

$$A + M \rightarrow AM \text{ (rate constant: } f) \quad \ldots.(1)$$

$$AM + XP \rightarrow AXP + M \text{ (rate constant: } g) \quad \ldots.(2)$$

$$AXP \rightarrow A + X + PO_4 \quad \ldots.(3)$$

In the resting state, reaction (1) must be prevented from occurring, so as to allow the muscle to lengthen or shorten passively. This might be achieved either by inactivating the enzyme which catalyses reaction (1), or by stopping reaction (3), which would leave the A sites blocked by the attachment of XP molecules, or by some independent change in either the A or the M groups which prevents them from uniting. For the sake of definiteness, the second of these assumptions is made here. Activation of the contractile substance then consists in causing reaction (3) to take place; it will be assumed that so long as activation persists, this reaction occurs fast enough for it not to be a limiting factor. (Some reasons for assuming provisionally that the activation change, whatever it is, affects the actin filaments and not the myosin ones, are given on pp. 278, 301, and 305). Further, it is assumed for the sake of simplicity that the back reactions are negligible in all three cases.

The reason why the rate constant g has to be given a finite value for positive as well as negative values of x is that otherwise the side-pieces that happen to be attached when activation ceases (in the sense that no more new attachments are formed) would remain attached, and the muscle would remain in a state of partial rigor instead of returning to the resting condition. This overlap of f and g causes a steady turnover of attached sites even when the muscle is stimulated isometrically; the resulting evolution of heat corresponds to HILL's "maintenance heat."

It is provisionally assumed that the sites on each filament are far enough apart for the events at one site not to be affected by the situation at nearby sites. There is no need to assume that the distance

apart of the M sites on each myosin filament is the same as that between A sites on an actin filament.

IV. 2. *Mathematical formulation*

Consider a large number of contraction sites all with A in the same position relative to O (Fig. 5), i.e. all having the same value of x. Let n be the proportion of these sites at which the M is combined with the corresponding A.

We have

$$\frac{\partial n}{\partial t} = (1 - n)f - ng$$

or

$$-v\frac{\partial n}{\partial x} = f - (f + g)n$$

where v is the velocity with which the A filament is sliding past the M filament as a result of the shortening of the muscle.

Now $v = sV/2$, where s is the sarcomere length and V is the rate of shortening in muscle lengths per second, so that

$$-\frac{sV}{2}\frac{\partial n}{\partial x} = f - (f + g)n. \qquad \ldots.(4)$$

To find the total rate at which chemical energy is being liberated, we note that the overall result of the reactions (1)–(3) is that one high-energy phosphate group is split off, liberating e ergs per contraction site in one cycle. The frequency with which A sites are presented to each M site is v/l, where l is the separation of the A sites along the actin filament. The average number of times each M site enters the cycle of reactions per second is therefore

$$\frac{v}{l}\int_{x=\infty}^{x=-\infty} f(1 - n)\mathrm{d}t, \quad \text{or} \quad \frac{1}{l}\int_{-\infty}^{\infty} f(1 - n)\mathrm{d}x.$$

The total rate of energy liberation per cubic centimetre of muscle, E, is therefore given by:

$$E = \frac{me}{l}\int_{-\infty}^{\infty} f(1 - n)\mathrm{d}x \qquad \ldots.(5)$$

where m is the number of M sites per cubic centimetre of muscle.

To find the tension in the muscle, assume that the elastic element obeys HOOKE's law with a stiffness k dyne/cm. The average value of the work done at one myosin site as one actin site is carried past it by the shortening of the muscle is then

$$\int_{-\infty}^{\infty} nkx\,\mathrm{d}x,$$

and the corresponding average force is obtained by dividing this by l.

The total tension in the muscle is the sum of the tensions generated by all the contraction sites within one half-sarcomere; the number of these sites for a muscle of 1 cm² cross-sectional area is $ms/2$. Hence P, the tension per square centimetre, is given by:

$$P = \frac{msk}{2l}\int_{-\infty}^{\infty} nx\,\mathrm{d}x. \qquad \ldots.(6)$$

The relations (4), (5) and (6) do not depend on the particular assumptions that are being made concerning the manner in which f and g vary with x; to introduce these assumptions, we must insert the values of f and g which are indicated in Fig. 6, i.e.

$$\begin{aligned} x < 0: &\qquad f = 0 \text{ and } g = g_2; \\ 0 < x < h: &\qquad f = f_1 x/h \text{ and } g = g_1 x/h; \\ x > h: &\qquad f = 0 \text{ and } g = g_1 x/h. \end{aligned}$$

Solving equation (4) with these values, and taking V as positive (i.e. the muscle is shortening, not being stretched) we find:

$$x > h: \qquad n = 0$$

$$0 < x < h: \qquad n = \frac{f_1}{f_1 + g_1}\left(1 - \mathrm{e}^{\left(\frac{x^2}{h^2} - 1\right)\frac{\phi}{V}}\right) \qquad \ldots.(7)$$

$$\text{where } \phi = (f_1 + g_1)h/s$$

$$x < 0: \qquad n = \frac{f_1}{f_1 + g_1}\left(1 - \mathrm{e}^{-\frac{\phi}{V}}\right)\mathrm{e}^{\frac{2xg_2}{sV}}. \qquad \ldots.(8)$$

These relationships between n and x are plotted, over the whole range, in Fig. 7 for four values of V. For reasons which will appear later, $g_1/(f_1 + g_1)$ is taken as 3/16 and $g_2/(f_1 + g_1)$ as 3·919.

Inserting these formulae in equation (5) and evaluating the integral, we obtain:

$$E = me \cdot \frac{h}{2l} \cdot \frac{f_1}{f_1 + g_1}\left\{g_1 + f_1 \frac{V}{\phi}\left(1 - \mathrm{e}^{-\frac{\phi}{V}}\right)\right\}. \qquad \ldots.(9)$$

When $V = 0$, this gives the "maintenance heat" rate, E_0, as

$$me \cdot \frac{h}{2l} \cdot \frac{f_1 g_1}{f_1 + g_1},$$

while the "extra rate of energy liberation" $E'(= E - E_0)$ is

$$E' = me \cdot \frac{h}{2l} \cdot \frac{{f_1}^2}{f_1 + g_1} \cdot \frac{V}{\phi}\left(1 - \mathrm{e}^{-\frac{\phi}{V}}\right). \qquad \ldots.(10)$$

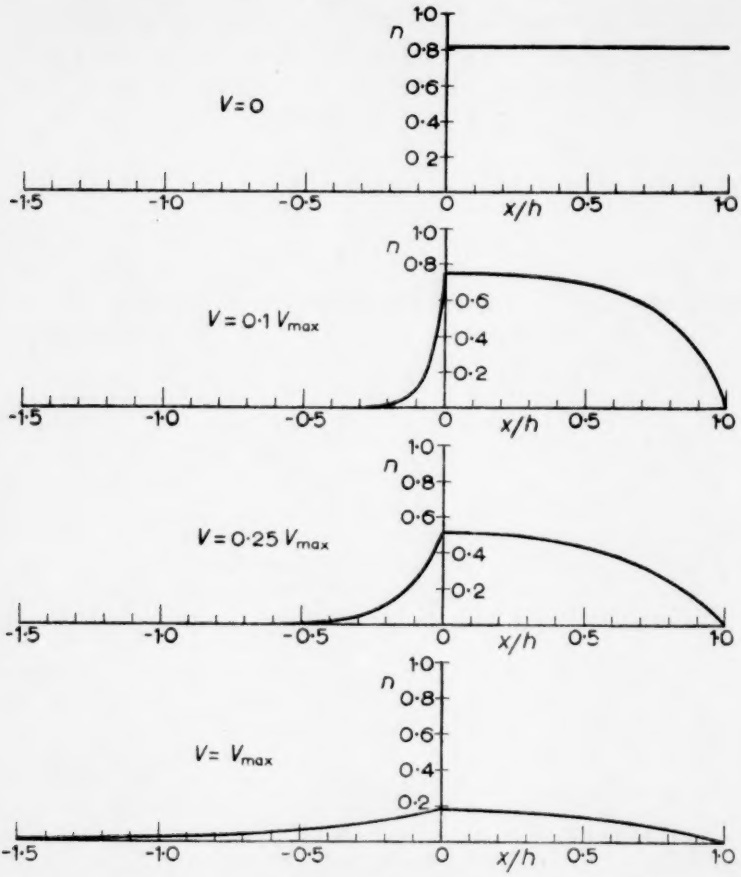

Fig. 7. Variation of n (proportion of sites at which links between actin and myosin are in existence) with x (position of A site relative to equilibrium position of M site), for the steady state in isometric contraction (*top*) and in shortening at three different speeds.

Similarly, equation (6) for the tension becomes:

$$P = \frac{msk}{2l} \cdot \frac{f_1}{f_1 + g_1} \cdot \frac{h^2}{2} \left\{ 1 - \frac{V}{\phi} (1 - e^{-\frac{\phi}{V}}) \left(1 + \frac{1}{2} \left(\frac{f_1 + g_1}{g_2} \right)^2 \frac{V}{\phi} \right) \right\}.$$

The maximum work done in a cycle at one site is $kh^2/2$; denoting this by w, we have:

$$P = \frac{msw}{2l} \cdot \frac{f_1}{f_1 + g_1} \cdot \left\{ 1 - \frac{V}{\phi} (1 - e^{-\frac{\phi}{V}}) \left(1 + \frac{1}{2} \left(\frac{f_1 + g_1}{g_2} \right)^2 \frac{V}{\phi} \right) \right\}, \quad \ldots . (11)$$

and the rate of doing mechanical work is

$$PV = mw \cdot \frac{hf_1}{2l} \cdot \frac{V}{\phi}\left\{1 - \frac{V}{\phi}(1 - e^{-\frac{\phi}{V}})\left(1 + \frac{1}{2}\left(\frac{f_1 + g_1}{g_2}\right)^2 \frac{V}{\phi}\right)\right\}. \quad \ldots.(12)$$

The rate of liberation of heat is $E - PV$.

IV. 3. *Comparison with Hill's equations*

To find out whether equations of these forms can represent the actual behaviour of muscle, we may see how closely they can be made to fit the relationships shown by A. V. HILL (1938) to be obeyed by frog muscle during tetanic stimulation at 0°C. These are:

(1) The rate of liberation of heat increases linearly with speed of shortening (constancy of shortening heat).

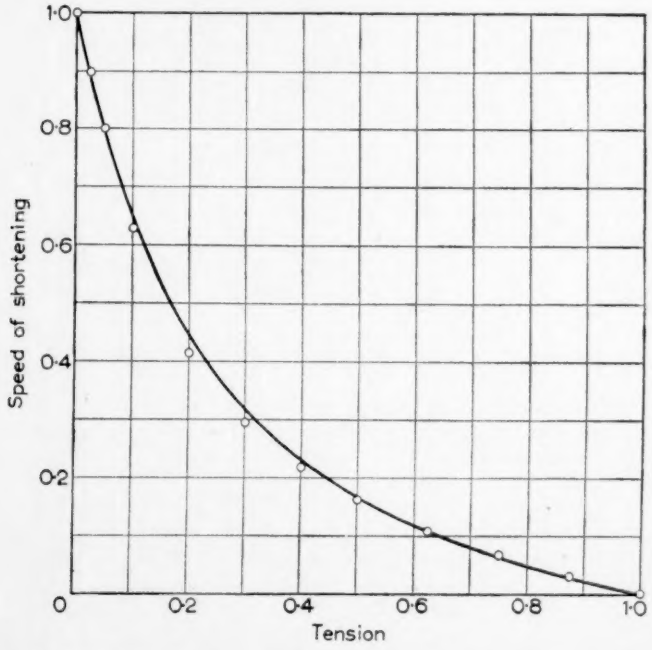

Fig. 8. Relationship between speed of shortening and tension. Continuous line: HILL's characteristic equation with $a/P_0 = \frac{1}{4}$. Circles: equation (11). Ordinate, speed of shortening as a fraction of value in unloaded tetanus; abscissa, tension as a fraction of value in isometric tetanus.

(2) The total rate of energy liberation (heat + work) increases linearly as the load is reduced below the isometric tension.

By combining these relationships HILL obtained his "characteristic equation," $(P + a)(V + b) = \text{constant}$, which he found to agree

excellently with the directly determined force-velocity curve. The constant a was found to be approximately one-quarter of P_0, the isometric tension; b is necessarily equal to a/P_0 multiplied by the speed of shortening under zero load.

Inspection of equations (9) and (12) shows that we have only two adjustable constants left for fitting our equations to HILL's relationships (with $a/P_0 = \frac{1}{4}$). They are

$$\left(\frac{w}{e}\frac{f_1 + g_1}{f_1}\right) \quad \text{and} \quad \frac{f_1 + g_1}{g_2}.$$

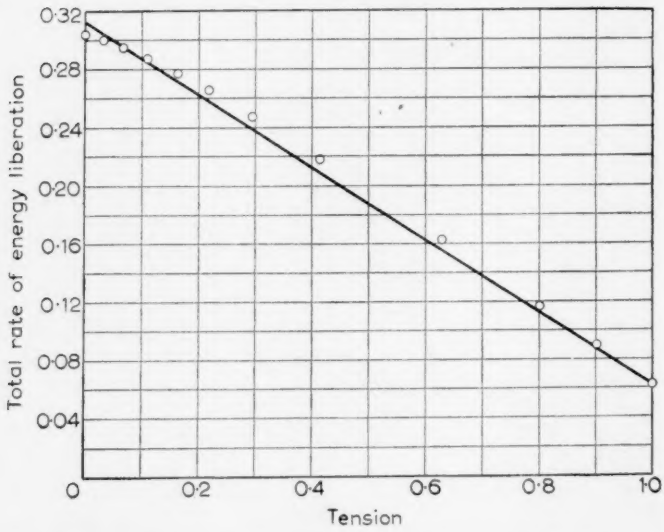

Fig. 9. Relationship between total rate of energy liberation (heat + work) and tension. Straight line, from HILL's equations with $a/P_0 = \frac{1}{4}$ and maintenance heat rate $= ab$. Circles: from equations (9) and (11). On abscissa scale, unity is P_0, the isometric tension; on ordinate scale, unity is the product $P_0 V_{max}$.

The ratio g_1/f_1 has also to be chosen so as to give the maintenance heat rate a value corresponding to that found experimentally; HILL found that it was about equal to ab.

Trial and error showed that fair agreement could be obtained by making $w/e = 0{\cdot}75$, $g_1/(f_1 + g_1) = 3/16$, and $g_2/(f_1 + g_1) = 3{\cdot}919$. These give $V_{max} = 4{\cdot}0\,.\,\phi$, so that ϕ is equal to HILL's b; they also make the maintenance heat rate bear the correct proportion (1/16) to the product $P_0 V_{max}$. The degree of success in matching HILL's relationships can be seen from Figs. 8, 9, 10, and 11. The deviations from hyperbolae in Figs. 8 and 11, and from straight lines in Figs. 9 and 10, are probably not much greater than the experimental error of the observations on which HILL based his relationships.

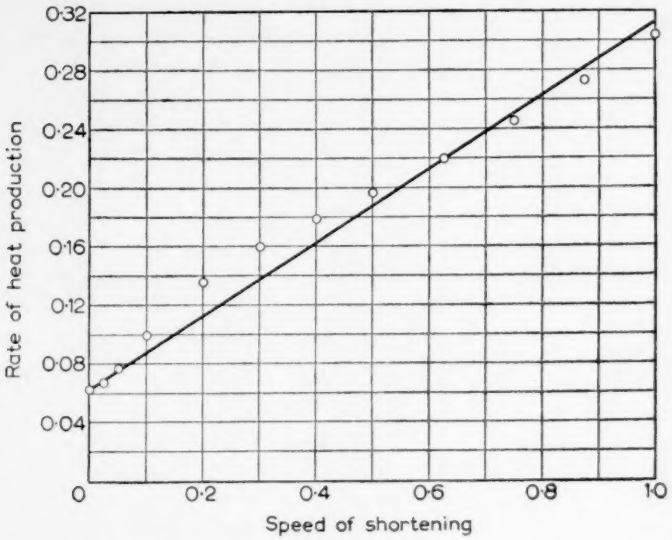

Fig. 10. Relationship between rate of heat production and speed of shortening. Straight line corresponds to a constant heat of shortening (as found by HILL) with $a/P_0 = \frac{1}{4}$ and maintenance heat rate $= ab$; circles from equations (9) and (12). Ordinate scale as in Fig. 9; unity on abscissa scale is V_{max}, the speed of shortening in an unloaded tetanus.

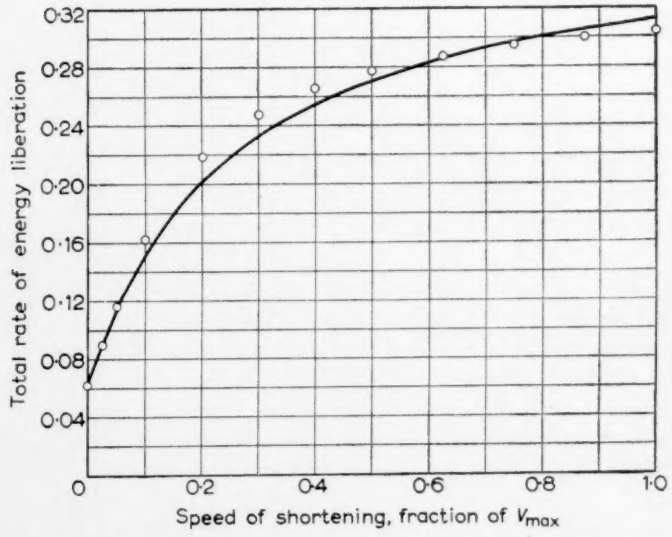

Fig. 11. Relationship between total rate of energy liberation and speed of shortening. Continuous curve (hyperbola) derived from HILL's relationships, with $a/P_0 = \frac{1}{4}$ and maintenance heat rate $= ab$; circles from equation (9). Ordinate scale as in Fig. 9.

Although only two constants were available for fitting HILL's equations, and one for fitting the amount of maintenance heat, arbitrary assumptions had been made at an earlier stage in making f and g vary with x in the ways shown in Fig. 6. One or two other forms of these relationships were also tried. With f and g constant (instead of increasing linearly with x) in the range $0 < x < h$, HILL's equations could be fitted about as well as with the system discussed here, but the ratio w/e had to be given a value of about unity. This is not plausible, as the three reactions that are postulated cannot all go spontaneously in the forward direction unless w is considerably less than e (which is assumed to be derived wholly from a change in free energy). This result was not much altered when the change-over from high f to high g was shifted from the point $x = 0$ to $x = h/5$. It can be seen intuitively that, for a given value of w/e, the efficiency will be lower in these systems with constant f than in the one developed here with f proportional to x, because during shortening a higher proportion of the attachments will be formed at small values of x (therefore contributing little to the work done) in the former system than in the latter.

Another system, within the same general framework, which is of interest, is one in which f and g vary exponentially with x. The equations are:

$$x < h: \quad f = f_{\max} e^{(x-h)/\lambda}$$

$$g = f_{\max} (1 - e^{(x-h)/\lambda})$$

$$\text{Force in elastic element} = p = p_{\max} \frac{e^{(x-h)/\lambda} - \alpha}{1 - \alpha}$$

$x > h$: $f = 0$, so that no combination occurs during shortening; g and p therefore need not be specified.

The interesting point about this system is that it satisfies HILL's equations exactly. Unfortunately it gives about 5 times too high a value for the maintenance heat, because of the great overlap between the functions defining f and g. Mathematically, this can be overcome if g is allowed to become negative when x has values near to h, but this is not permissible, at any rate without extra assumptions, as it implies the spontaneous reversal of a reaction which goes in the forward direction with the release of a large amount of free energy. For this reason, I believe that it is unprofitable to search further for schemes, within the framework of the general hypothesis that is under discussion, which obey HILL's equations exactly, since this particular system is probably unique in giving an exact agreement, apart from a "family" of systems that can be obtained from it by transformations in which the ratios between f, g, and p are unchanged at each value of x, and which lead to the same relations between speed of shortening, tension, and rate of liberation of heat.

IV. 4. *Consequences of the hypothesis*

IV. 4. 1. *Lengthening of a stimulated muscle*

Several interesting results come out if one follows up the consequences of this hypothesis. The first that I shall deal with concerns the tension in the muscle when it is forcibly stretched during tetanic stimulation. HILL (1938) found that there is a discontinuity in the force-velocity curve at the point where the velocity reverses: the increase of tension, above isometric, required to produce a given small speed of lengthening is much greater than the drop in tension which allows an equal speed of shortening. KATZ (1939) investigated this further and his curves (fig. 5) show that $-\mathrm{d}P/\mathrm{d}V$, the slope of the force-velocity curve, is about six times greater for slow lengthening than for slow shortening. Our hypothesis leads to a similar discontinuity, because during slow lengthening n falls off beyond $x = h$ with a space constant equal to $(-)v/g_1$, while during shortening the corresponding space constant of the deficit of n below its steady-state value is $v/(f_1 + g_1)$, which is several times smaller. The resulting changes in tension are proportional to these space constants, but during lengthening there is also a drop in tension due to the fall of n near $x = 0$. The overall result is that the ratio of values of $\mathrm{d}P/\mathrm{d}V$ for slow lengthening and slow shortening is f_1/g_1, which we have taken as 4·33. This figure is to be compared with KATZ's value of about 6.

As the speed of lengthening is increased, n becomes appreciable at greater and greater values of x, and we are assuming that g rises in proportion. Hence, the dissociation of AM links becomes more rapid, while on the other hand there is less time for the links to be formed because of the greater speed. Together these two factors set a limit to the rise of tension; as the speed is increased indefinitely the tension approaches asymptotically a value $P_0(f_1 + g_1)/g_1$, or $5{\cdot}33P_2$ with the values taken here. This is qualitatively similar to the "give" or "slip" which happens when a muscle is stretched during a tetanus, at anything but a very low speed (HILL, 1938), but KATZ (1939) found that the load needed to cause very rapid lengthening was only $1{\cdot}8\ P_0$.

A qualitative explanation is also given for another phenomenon described by KATZ (1939, fig. 7). A muscle is tetanized and allowed to shorten under small load until it reaches a stop; then, before it has had time to develop much tension, a load about equal to the isometric tension at the shortened length is applied. The muscle is considerably stretched, and then shortens again to approach the stop asymptotically. The explanation would be that during the initial rapid shortening the total number of links in existence is small, as shown in Fig. 7 (p. 286). If the load is applied before the number of links has had time to increase, it will be more than they can hold, and the muscle will "give."

A limit to the quick stretch is presumably set by the fact that the muscle is being brought to greater lengths, at which the isometric tension of which the muscle is capable is also greater.

So far, the system has accounted well for the behaviour of a muscle that is stretched during stimulation. But as it stands it does not predict correctly the amount of heat that is liberated. ABBOTT, AUBERT and HILL (1951) found that during slow lengthening the rate of evolution of heat was increased by an amount which was less than the equivalent of the work done on the muscle; ABBOTT and AUBERT (1951) found that the total rate of evolution of heat, including the heat derived from the work done, might even be less than the maintenance heat rate during an isometric tetanus. On the system we are considering, the rate of evolution of heat (apart from mechanical work done) should increase with speed of lengthening in exactly the same way as the total rate of energy liberation increases with speed of shortening (equation 9). This discrepancy could be eliminated, at least qualitatively, if it were assumed that at the larger values of x the AM links were broken not by reaction (2) (p. 283) but by the reversal of reaction (1), which does not involve the splitting of a high-energy phosphate bond. There is no difficulty in principle about this reversal, of the kind that prevented us from assuming a reversal of reaction (2); indeed the equilibrium of reaction (1) is bound to go in favour of the dissociated state at sufficiently large values of x because of the large amount of energy needed to bring M to the position where combination can occur, and the consequent rarity of collisions between A and M.

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IV. 4. 2. *Unloaded shortening*

It has been assumed tacitly that the only factors limiting the speed of shortening are the applied load and the resistance generated by those links which are still connected after x has become negative. For a given speed of shortening, the second of these terms will be proportional to the number of sites which are activated, as also is the tension generated by the contraction process. If the load is zero, therefore, the tension generated and the internal resistance to shortening will become equal at a speed of shortening which is independent of the number of sites that are active; in other words, the speed of unloaded shortening is independent of the degree of activation. In the real case, no doubt there are other kinds of resistance to shortening which do not decrease proportionately with the degree of activation, so that the speed of shortening would fall off when the degree of activation becomes very small, but nevertheless this argument does show that the independence might hold, within experimental error, over a large part of the range. This result, which would probably be true also for many other types of contractile system, suggests that

caution is necessary in interpreting the rapidity with which speed of shortening reaches its maximum after a stimulus as evidence that activation is complete equally early (HILL, 1951a; ABBOTT and RITCHIE, 1951b).

IV. 4. 3. *Activation and relaxation*

The concepts of active state and degree of activation, introduced by A. V. HILL, have been very fruitful in reducing a wide range of phenomena in muscle to dependence on a single variable. A muscle is said to be in the active state when it is capable of shortening or developing tension; during full activation it obeys HILL's characteristic equation. The degree of activation at any instant is measured by the tension which the muscle can just hold without either shortening or lengthening (HILL, 1949c). HILL and others have shown that to a large extent the time course of tension in a twitch can be explained by supposing that the degree of activation rises from zero to its maximum very quickly after the stimulus, stays at its maximum for an appreciable time, and then falls away gradually to zero. The relatively slow rise of tension, and the failure of tension to reach, in a twitch, the value that it achieves in a tetanus, are explained by the presence of a series elastic element which the contractile elements have to stretch by a finite amount in order for the tension to appear at the tendon. Relaxation is explained simply as the disappearance of this active state (HILL, 1953b).

On the hypothesis that we are discussing here, activation in this sense involves two distinct steps. The first consists in allowing reaction (3) (p. 283) to occur; this reaction is the removal of the phosphate groups which during the resting state have been preventing the A elements from combining with M. The second step is the actual formation of the A–M links. Nothing has been said so far about reaction (3) except that during a fully developed tetanus it is so fast as not to be a limiting factor. If we assume further that it is complete practically instantaneously after a stimulus, there will still be delay in the development of the degree of activation as defined by HILL, because the muscle can only hold tension in proportion to the number of AM links that have been formed, and the rate of formation of these links is set by the rate constant f which is finite and is related to the maximum speed of shortening of the muscle. The same distinction arises in relaxation: even if the degree of activation fell to zero suddenly because reaction (3) was stopped, the AM links already in existence would persist for a finite time, their number decaying with the rate constant g. So long as any of these links persisted at the end of an isometric twitch, there would still be some tension, but the muscle would not be capable of continued shortening if the load was removed because no new AM links could be formed.

Reaction (3) was chosen somewhat arbitrarily on p. 283 as the one that was inhibited during the resting state, and it was mentioned there that inhibition of reaction (1), the actual formation of the links, might equally well be the means by which relaxation was produced. Even if this was so, there would still be two steps in activation, namely the removal of this inhibition and the formation of the links: even if the first of these was instantaneous, the second would only take place with the rate constant f. Short of postulating a dual nature for the initial step, I have not been able to think of any variant of the general hypothesis which would not give two stages in both activation and relaxation.

If, then, two-stage activation and relaxation are necessary consequences of the hypothesis, it is natural to ask whether there is any experimental evidence for either or both of these phenomena. One case where such evidence exists is in the indirect flight muscles of certain insects and the tymbal muscle of cicadas, where PRINGLE (1949, 1954) has shown clearly that two distinct kinds of activation are involved. In their normal activity *in situ*, these muscles contract in an oscillatory manner at a frequency very much higher than the frequency of the action potentials in their fibres. In the cicada muscle, PRINGLE shows that a single action potential produces an active state lasting between 50 and 100 msec, which can summate with the effects of succeeding stimuli. When the muscle is attached to a lever for recording its contraction, an ordinary twitch or tetanus results, but if it is *in situ*, it performs a series of contractions at intervals of 3–10 msec so long as the active state is at a sufficient level. Each of these contractions causes the tymbal (the "drum" with which the cicada makes its song) to click from an "out" to an "in" position; about 1 msec after this contraction the tymbal clicks out again, stretching the muscle. Clearly, some kind of de-activation must have occurred as a result of the shortening, which allows the outward click to occur; this must last for only a very short time as the muscle contracts again a few milliseconds after the outward click and repeats the cycle; up to 8 of these cycles may occur within 40 msec or so after a stimulus which produces only a single action potential. On our hypothesis, the effect of the action potential might be to remove the inhibition of reaction (3) for say 50 msec, while shortening might cause dissociation of the AM links, which would then need a certain time to reform. The important point, however, is that these muscles—admittedly very specialized ones—show two quite distinct kinds of activation.

In ordinary muscle it might be expected that, if these two stages exist, they would be easier to distinguish during relaxation than at the beginning of a contraction, since it was shown above that the time constant for breaking the AM links in relaxation ($\simeq 1/g$) should

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be several times longer than the time constant for forming them at activation ($\simeq 1/f$). In an isometric twitch, this two-stage relaxation would show up by the active state (detected for instance by the ability of the muscle to redevelop tension after a quick release) falling to zero while there was still considerable tension remaining. This point does not seem to have been submitted to direct experimental test, but several published results suggest that muscles do behave in this way. For example, in the contraction shown in fig. 1 (top curve) of a paper by RITCHIE and WILKIE (1955) the degree of activation has fallen to 7 per cent of its maximum value at 330 msec after the stimulus, while the tension is still 90 per cent of its value at the peak of the twitch, and 74 per cent of the isometric tetanus tension.

It also seems difficult to explain some of the quick stretch phenomena described by KATZ (1939) without distinguishing between two steps in the activation process (cf. pp. 291 and 308).

IV. 5. *Size and number of contraction sites*

IV. 5. 1. *Range of movement of side-pieces*

A rough estimate of the value of h, the largest displacement at which a side-piece can become attached to an actin filament, may be obtained as follows. On p. 288 it was shown that the quantity $\phi(= (f_1 + g_1)h/s)$ came out to be equal to HILL's b; hence

$$h = bs/(f_1 + g_1). \qquad \ldots.(13)$$

Now $g_1/(f_1 + g_1)$ was set at 3/16, in order to give the right amount of maintenance heat; hence $h = 3bs/16g_1$. b and s are known (for frog muscle at 0°C), and an estimate for g_1 can be obtained from the decay of tension at the end of an isometric twitch, accepting provisionally the interpretation given in the last section. The same curve in RITCHIE and WILKIE's paper that was referred to in that connection gives the time constant of decay of tension as about 150 msec at about 500 msec after the stimulus, when the degree of activation appears to have fallen to zero. This time constant should be of the order of $1/g_1$; this is not an exact equality because g varies with x (so that the theoretical time course is not exponential), and only reaches g_1 when $x = h$. Hence $1/g_1$ is likely to be less than 150 msec; we might take 100 msec, making $g_1 = 10\ \text{sec}^{-1}$. Now HILL (1938) found that b is about $\frac{1}{3}\ \text{sec}^{-1}$, and s is about 2·5 μ; inserting these values in the above equation we obtain

$$h = \frac{3 \times \frac{1}{3} \times 2{\cdot}5}{16 \times 10}\,\mu = 156\ \text{Å}.$$

This figure is subject to considerable uncertainties. Thus, the values taken for b and for g_1 were obtained from different frogs, and the

estimate of g_1 was in any case very rough; also the value for the ratio f_1/g_1 was obtained on the somewhat arbitrary assumptions (*a*) that the whole of the maintenance heat is derived from the breaking of AM links at values of x between 0 and h, and (*b*) that no links are broken by a reversal of reaction (1). None the less, the value is one that would fit in well with the attractive possibility that the side-pieces are placed at intervals along the filaments equal to the 415-Å period which has been observed with X-rays (BEAR, 1945; H. E. HUXLEY, 1952, 1953b) and in the electron microscope (HALL, JAKUS and SCHMITT, 1946; DRAPER and HODGE, 1949).

IV. 5. 2. *Distance between successive A sites*

On p. 286, a formula for the tension during tetanic stimulation was derived (equation 11). Putting $V = 0$, we obtain for the isometric tetanus tension

$$P_0 = \frac{msw}{2l} \cdot \frac{f_1}{(f_1 + g_1)}. \qquad \ldots.(14)$$

w was set at $\frac{3}{4}e$, and $f_1/(f_1 + g_1)$ at 13/16, so that

$$l = 0{\cdot}305\, mse/P_0. \qquad \ldots.(15)$$

Values for the quantities on the right-hand side of this equation may be obtained as follows. s is $2{\cdot}5\ \mu$ or $2{\cdot}5 \times 10^{-4}$ cm; P_0 for frog muscle is about 2 kg/cm², or 2×10^6 dyne/cm². m, the number of M sites per cubic centimetre of muscle, may be provisionally identified with the number of myosin molecules in the same volume; this may be calculated from a concentration of 8 g/100 ml. and a molecular weight of 840,000 (WEBER, 1950) to be $5{\cdot}7 \times 10^{16}$/c.c. If the heat of hydrolysis of the high-energy phosphate group is taken as 10 kcal/mole, then e is 7×10^{-13} erg/molecule. Inserting these values, we obtain $l = 153$ Å.

The interpretation of this result is complicated by the fact that it is close to the estimate we have just obtained for h. Equation (6), from which equations (11) and (15) were derived, assumes tacitly that l is considerably greater than h, so that each M site is always free from its last attachment to an A before the next A presents itself for combination. If l was really smaller than h, the calculation we have gone through would probably give the value of h, not of l; the relationships between force, velocity and heat production would also be modified if l was not appreciably greater than h. We may conclude either that l is equal to or smaller than h, in which case the figure we have obtained is a confirmation of the other estimate of h but the formulae for the force-velocity relation, etc. are no longer exactly appropriate, or else that l is about 150 Å and h is appreciably less, say 100 Å, which would

probably not be too small a value to conflict seriously with the earlier estimate.

IV. 5. 3. *Spacings expected on structural grounds*

Estimates of the spacings of myosin and actin molecules along their respective filaments can be obtained from the quantities of the proteins present, their molecular weights, and the arrangement and spacing of the filaments deduced from H. E. HUXLEY's X-ray and electron-microscope observations. These calculations have been made by HANSON and H. E. HUXLEY (1955, p. 253). For the myosin filaments, assuming that there are six molecules abreast (one facing each of the six actin filaments which surround the myosin filament), they find that the longitudinal spacing comes out to about 400 Å, agreeing remarkably with the well-known 415-Å period seen with X-rays and the electron microscope. Each actin filament is surrounded by three myosin filaments; assuming therefore three actin molecules abreast, they calculate a longitudinal spacing of about 130 Å. This is close to the upper limit that was obtained in the last section for the distance between successive *A* sites with which any one *M* site can combine; it would be very natural to identify this with the spacing of actin molecules along a filament.

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IV. 6. *Discussion*

At the outset, it must be emphasized that the agreement which has been achieved with some aspects of the known behaviour of muscle is not to be regarded as grounds for accepting the scheme which has been put forward. There is little doubt that equally good agreement could be reached on very different sets of assumptions, all equally consistent with the structural, physical, and chemical data to which this set has been fitted. The agreement does however show that this type of mechanism deserves to be seriously considered, and that it is worth looking for direct evidence of the side-pieces, and of the localization of enzymic activity, which have been postulated.

Quite apart from the possible value of this scheme as a working hypothesis, several of the results are of more general interest. The proposed mechanism may be described as cyclic, in the sense that the number of sites in a given condition is not affected by shortening: each side-piece goes through cycles in which it combines with the actin filament by one reaction and is separated from it by another. The final states of the side-piece and of the site to which it was attached are the same as their initial states; the only changes are that the muscle has shortened, an energy-rich phosphate bond has been split and work may have been done. The mechanism may be contrasted in this respect with any of the theories that postulate folding links in series: in these

the number of links in the folded condition increases as the muscle shortens. Mechanisms based on the sliding hypothesis are not necessarily of the cyclic type: of the three classes of mechanism defined on p. 279 (2) and (3) are cyclic but (1) is not.

In a cyclic system, work may be done several times at each site during a given contraction, while in other systems net work can only be done once. If the work per cycle at one site in the first case is of the same order of magnitude as the work per link folded in the second (e.g. because both are related to the free-energy release on hydrolysis of an energy-rich phosphate bond) the number of sites per unit volume will come out smaller in the cyclic systems than in others. This is illustrated by the fact that the molecular weight of the active unit of myosin is assumed here to be 840,000, while both SZENT-GYÖRGYI (1953, p. 36) and POLISSAR (1952d), assuming non-cyclic systems, arrive at figures of around 40,000.

Another point is that several of the general questions that have been asked about muscle (for instance, whether energy-rich phosphate is split during contraction or during relaxation) may lose their meaning in connection with cyclic mechanisms. Thus, in the system developed here, phosphate is split off, by the same reaction, during both contraction and relaxation. Similarly, the question discussed by MORALES *et al.* (1955), whether contraction is produced by the combination or by the splitting of ATP, has no answer on a system of the kind discussed here: the immediate cause of tension development is the actual formation of the links between actin and myosin.

In this system, as in POLISSAR's, HILL's relations between load, speed of shortening and heat production are brought out as approximations. This is perhaps rather unsatisfying, but it does act as a reminder that theories need not be discarded simply because they do not lead exactly to the mathematical formulation that was given by HILL, so long as they fit the experimental data adequately.

A feature of some general interest is brought out by considering the second of HILL's relationships (p. 287) in a slightly different form. It is usually expressed by saying that the total extra rate of energy liberation (in excess of the maintenance heat) increases linearly as the load is reduced below the isometric tension, being equal to $b(P_0 - P)$. If we substitute for P the value given by HILL's characteristic equation, this becomes $b(P_0 + a)V/(V + b)$. Thus, as the speed of shortening increases, the total rate of energy liberation rises rapidly at first and then more slowly (cf. Fig. 11). This shows directly that the amount of chemical change per unit amount of shortening is not a constant, but falls as the speed of shortening rises. D. M. NEEDHAM (1950, pp. 48–49) drew attention to this point, concluding that individual sites were active more than once during a contraction if it were carried

out at low speed, and that, even if ATP breakdown were associated with restoration of the protein chains, it might occur during the contraction phase, bringing the sites into a condition in which they could react again, as well as during relaxation. In the system described here, the fall of energy liberation for unit shortening as the speed is increased is provided for by giving a finite (as opposed to infinite) value to the rate constant f for the formation of the links: as the speed of shortening is increased, there is a parallel rise in the chance that a pair of A and M sites will pass each other without any chemical reaction taking place.

It is natural to ask whether the mechanism proposed here for striated muscle could account also for the contraction of smooth muscle. On general grounds, it is to be expected that the mechanism is fundamentally the same in both types, so that it would be unsatisfactory to postulate for one type a mechanism that clearly cannot exist in the other. Not enough is known at present about the submicroscopic structure of smooth muscle to make a definite statement either way, but there does not seem to be anything to exclude the possibility that smooth muscle contains, in a much less orderly arrangement, filaments which are moved past one another by a mechanism similar to that proposed here for striated muscle. The absence of any marked change in the wide-angle X-ray pattern (ASTBURY, 1947) and in the strength of the intrinsic component of the birefringence (FISCHER, 1944) when smooth muscle is stretched or shortened over a wide range, do indeed suggest that the filaments move relative to one another without much internal rearrangement; and as long ago as 1936, BOZLER suggested that the mechanical behaviour of smooth muscle could be more easily explained by assuming that contraction took place by relative movement between the molecules, than by changes in their shape.

V. OTHER PHENOMENA IN MUSCLE

The hypothesis of muscular contraction set out in the preceding sections was originally developed as an attempt to fit together the available information on (1) muscle structure, (2) the relationships between shortening, tension and heat liberation, and (3) the outstanding facts concerning the interactions of actin, myosin and ATP. There are of course many other phenomena which may provide important clues to the mechanism of contraction and which will have to be explained by any theory which aims at completeness. In the following paragraphs, some of these phenomena will be discussed in relation both to the idea that length changes take place by sliding of actin and myosin filaments past one another, and to the particular hypothesis which has been developed here. Possible new interpretations of the data emerge in several cases.

V. 1. *Early changes in a twitch*

Several physical changes are known to occur very early after a stimulus is applied to a muscle, all being apparent during the latent period before the rise of tension or shortening of the muscle begins, and being complete well before the peak of the twitch. The principal ones are the following.

(1) A small drop in tension (the "latency relaxation") precedes the main rise (RAUH, 1922; SCHAEFER and GÖPFERT, 1937; SANDOW, 1944, 1947).

(2) The torsional rigidity of an isolated fibre increases (STEN-KNUDSEN, 1953).

(3) An increase in the hydrostatic pressure to which the muscle is subjected causes an increase in the tension that is subsequently developed ("alpha process," BROWN, 1934, 1936, 1941).

(4) The resistance of the muscle to passive stretch begins to rise about half way through the latent period (A. V. HILL, 1950b, 1951b).

(5) The rate of heat production rises rapidly to its maximum about half way through the latent period (A. V. HILL, 1949b, 1950a, 1953a).

(6) The amount of light diffracted by the striations decreases (D. K. HILL, 1953).

Probably all of these changes begin at about the same time, but there is a good deal of uncertainty, partly because parallel measurements have been made in only a few cases (1 and 6, D. K. HILL, 1949; 4 and 5, A. V. HILL, 1950a,b), and partly because the time resolution is not good enough in some cases (2 and 3) for precise comparisons to be made. The later part of the time course is obscured by other changes which accompany the contraction itself, except in cases 2 and 3.

V. 1. 1. *Latency relaxation*

The amplitude of the early fall in tension, and the duration of the period in which the tension is below its resting value, are both found to increase as the muscle is stretched (SANDOW, 1944; ABBOTT and RITCHIE, 1951a). A. V. HILL (1951b) has pointed out that this is difficult to explain if it is assumed that the relaxation is due to a lengthening of a structure which is in series with the contractile element and with the "series elastic element" which limits the rate of rise of tension: if the increase in the amplitude of the latency relaxation were due to increased stiffness of the series elastic component, then the rate of rise of tension ought to be increased for the same reason, and the time at which the tension curve re-crossed the baseline ought to have been unchanged. The observation would however be easily explained if the relaxation was due to the lengthening of a structure in parallel with both the contractile and the series elastic elements. This was difficult to fit in with the accepted idea that the contractile material was continuous along

the fibre even at rest; almost the only remaining possibility was the rather improbable one that the latency relaxation took place in the sarcolemma and did not involve the contractile material at all. The difficulty disappears however if the sliding model is adopted. If it is supposed that some part of the resting tension is taken by the actin filaments and the *S* filaments by which they are joined, then the latency relaxation could be the result of a lengthening of the actin filaments (Fig. 1), which could allow the elastic *S* filaments to shorten, reducing their tension. If the tension in the *S* filaments behaves at all like the total resting tension (increasing roughly exponentially with stretch), the tension drop for a given elongation of the actin filaments will increase with muscle length. The *S* filaments are not stretched by the contractile process and therefore have nothing to do with the series elastic element; there is therefore no reason to suppose that the latter becomes less compliant with increase of muscle length.

On this argument, the latency relaxation should represent a lengthening of the actin filaments. There is at present no evidence for an elastic connection from the end of each myosin filament to the adjacent *Z* line, but equally there is nothing to exclude this possibility; if such a thing existed it would be possible for lengthening of the myosin filaments to produce a drop in tension, and the latency relaxation might represent a change in them and not in the actin filaments. In either case, however, it is necessary to assume that the lengthening takes place in one type of filament independently of the other, and does not involve for instance a relative force generated between the actin and myosin filaments.

In the sequence of events which follow stimulation of a muscle, the change which underlies the latency relaxation must come earlier than the contraction itself. It is therefore probably a link in the chain of events which leads to activation of the contractile mechanism, and if it is indeed a change in the actin filaments, it is natural that the structure which conveys activation inwards from the membrane should be located in the *I* bands, as appears to be the case (p. 275).

V. 1. 2. *Increase in torsional rigidity*

Sten-Knudsen (1953) found that the torsional rigidity of an isolated fibre of the frog begins to increase before the main rise of tension, reaching a plateau at about one-third the contraction time and maintaining this level until well into the relaxation phase. He very naturally explained this early rise as being due to an increase in the number of cross-links between longitudinal protein chains, but this introduces a difficulty if the myosin and actin are distributed in the way shown in Fig. 1. Cross-links would be expected to be formed only in the zone of overlap, and the low rigidity of the *I* and *H* bands would prevent

any large change from being detected at the tendon ends. With the fibre at "equilibrium length", where the actin filaments of the two ends of each sarcomere are just in contact at the middle of the *A* band, STEN-KNUDSEN found a 20-fold increase of torsional stiffness, while the largest effect that could be expected from increased rigidity in the *A* bands alone is a fourfold increase, since at this muscle length the *I* band forms about a quarter of the sarcomere. It is difficult to suggest what the nature of the change may be, especially as there is no evidence at present whether it arises within the fibrils, from connections between the fibrils (e.g. the *Z* membranes) or from sarcoplasmic structures.

V. 1. 3. *The "alpha process"*

BROWN (1934, 1936, 1941) showed that the application of a pressure of a few hundred atmospheres to a muscle during the early part of an isometric twitch would cause an increase in the peak tension and in the rate of rise of tension, even though the pressure had been reduced to normal by a tenth of the contraction time. BROWN's suggestion (1941), that the high pressure acts by favouring the alteration in chemical behaviour of the muscle that results from stimulation, appears to require that the degree of activation in HILL's sense should be increased, but this appears to conflict with the evidence (HILL, 1949c; MACPHERSON and WILKIE, 1954) that activation is complete even in a twitch at atmospheric pressure. A possible alternative explanation is suggested by the sliding mechanism, together with BROWN's further observation that the application of pressure later in a twitch (e.g. near the peak) causes a *sudden* fall of tension (as well as an accelerated decay if the pressure is maintained). This suggests that increased pressure causes lengthening of either the actin or the myosin filaments (or both); if this is so, then a high pressure at the time when links are first being formed between the actin and myosin filaments will cause the filaments to become united with a greater degree of overlap, and the release of pressure will act just like a quick stretch, which is known to cause an increase in the tension subsequently developed in the twitch by helping to stretch the series elastic elements (HILL, 1949c).

V. 1. 4. *Early decrease of extensibility*

A. V. HILL (1950b, 1951b), showed that, if a muscle is stimulated while it is being stretched, an increase of tension is detectable at a much shorter interval after the stimulus than during a twitch without stretching. This effect begins at about the same time as the latency relaxation, and it has been generally assumed that both are manifestations of the same underlying process. This does not fit in with the hypothesis developed earlier in this article, since the earliest connection between the actin and myosin filaments would generate tension, and

no change involving one type of filament only should be able to alter the extensibility of the muscle; it also does not fit with the idea that the latency relaxation is due to lengthening of filaments without interaction between the two types (p. 301). An alternative is to suppose that the tension-generating process (formation of links between actin and myosin) begins at a very low rate early in the latency relaxation. The amount of tension developed would at first be masked by the relaxation, which is occurring in parallel at the same time, but when the muscle is stretched, the decreased extensibility due to the links might cause the total tension to rise instead of fall. That such an overlap is possible is shown by SANDOW's (1947) analysis of the time course of the early tension changes, in which the rate of rise of the component of tension generated by the contractile process itself was taken to be proportional to the extent of the lengthening which produces the latency relaxation, and a very good fit was obtained with the overall tension change.

V. 1. 5. *Early heat liberation*

A. V. HILL (1949a) has suggested that the heat liberated in a twitch consists only of activation heat and shortening heat, there being no component equivalent to the maintenance heat of a tetanus, which he regards as composed of the summed elements of activation heat that are liberated in response to the successive stimuli in maintaining the state of activation. In a sense, the scheme put forward here (pp. 279–284) fits in with this, in that both activation and maintenance heat must arise at least in part from the same reactions, but as far as that goes, shortening heat also arises from the same reactions. It would seem more natural, if that scheme is correct, to regard maintenance heat as something distinct from activation heat both in a twitch and in a tetanus, the activation component being associated only with the initial decrease in the number of sites blocked by combination with XP (p. 283). The rate of liberation of activation heat would then be proportional to the rate at which sites are being converted to the state in which the links between actin and myosin can be formed. The actual formation of the links is further delayed because it takes place with a finite rate constant (f, p. 281), so that the degree of activation, in the sense of the ability to hold tension, which must be proportional to the *number* of links in existence, lags by two steps behind the *rate* of liberation of activation heat. On this basis, the early maximum in the heat rate is not direct evidence that activation is complete very soon after the stimulus. The activation heat may also contain a component corresponding to the formation of the links between actin and myosin, but this component could not, on this formulation, contribute appreciably to the early peak.

V. 1. 6. *General discussion of early changes*

The discussion in the last few sections has led to postulating three steps in the activation process, apart from membrane changes and the inward spread along the Z membranes. These steps, which agree rather closely with those postulated by SANDOW (1947) in order to account for the early tension changes, are:

(1) Some changes which allows the reaction $AXP \rightarrow A + X + PO_4$ to take place (reaction 3, p. 283).

(2) The reaction $AXP \rightarrow A + X + PO_4$ itself; this reaction is accompanied by the liberation of "activation heat" and by the lengthening whose early stages show up as the latency relaxation.

(3) The formation of the links between actin and myosin by which tension or shortening is generated. The "alpha process" would correspond probably only to the earlier part of this step: as more links are formed, an increase of pressure would be progressively less able to cause the relative motion between the two sets of filaments on which it was assumed (p. 302) that the effect depends. The increase of torsional rigidity would presumably also correspond to this step.

Step (1) must proceed with a time constant of one or two milliseconds in frog muscle at 0°C, since the rate of step (2), as indicated by heat production, reaches its maximum in a time of this order (HILL, 1949b, 1953a).

The time constant of step (2) might be set equal to that of the early fall in the heat rate (after shortening heat has been deducted); from A. V. HILL's work (1949c) this appears to be roughly 25 msec. This may well be an overestimate since the activation heat may contain an element corresponding to step (3).

According to the hypothesis developed on pp. 281–288, the rate constant for step (3) is $(f + g)$. This varies according to the relative positions of the reactive sites; an average value for g was taken on p. 295 as 6·7 sec^{-1}, and $(f + g)/g$ was given the value 5·33, so that $(f + g)$ would be 35·6 sec^{-1}, corresponding to a time constant of 28 msec.

These time constants for steps (2) and (3) are about right to account for the rise of torsional rigidity (assumed to correspond to step 3) being complete at about 100 msec after the stimulus (all times refer to frog muscles at 0°C).

V. 2. *Decreased extractability of proteins*

It has been known for a long time that the fibrillar proteins of muscle are much more easily got into solution from fresh muscle than from muscle in fatigue or rigor (Deuticke-Kamp effect). At the same time a new component appears in the extracts (contractine, DUBUISSON, 1950).

BANGA and SZENT-GYÖRGYI (1943) showed that the decrease of extractability is largely explained by the union of actin and myosin, supposed to be separate in the resting fibre, to form actomyosin, but the observations of HASSELBACH (1953) suggest that other factors may also be involved. He found that a solution of ionic strength 0·6, containing pyrophosphate, will extract the myosin from minced muscle, leaving filaments presumably of actin but removing the *Z* lines. If the residues are broken up with a blender in the same solution, the actin is dissolved as well (HASSELBACH and SCHNEIDER, 1951); similarly, this solution dissolves both the actin and the myosin from fibrils prepared from fresh muscle (PERRY, 1955). On the other hand, fibrils prepared from muscle in rigor mortis lose only their myosin on extraction with this solution (HASSELBACH, 1953), the actin filaments and the *Z* lines being retained; fibrils from glycerol-extracted muscle appear to behave in the same way (HANSON and H. E. HUXLEY, 1953). Thus, both the *Z* lines and the actin filaments are protected by rigor from being dissolved, while the actin filaments, but not the *Z* lines, are preserved in muscles which has been minced but not broken up into fibrils. Clearly some change has taken place in the material of the *Z* line on rigor. Possibly this is enough to explain also the increased resistance to extraction of the actin filaments, or an alteration may have taken place in them too, which is an interesting possibility in connection with an earlier suggestion that the actin filaments may undergo a lengthening when the muscle is activated (p. 301).

The existence of a change in the physical properties of the *Z* line material further suggests that changes in this structure may conceivably be involved in some of the mechanical accompaniments of activation (e.g. the increase in torsional rigidity). On the other hand, it also suggests that rigor mortis involves definite activation of the contractile substance (since the function of the *Z* line appears to be to transmit activation) and not merely a union between actin and myosin resulting directly from the reduced ATP concentration. Suggestions that activation occurs in other kinds of rigor have recently been put forward on other grounds (SANDOW and SCHNEYER (1955) for iodoacetate rigor, and BARNES, DUFF and THRELFALL (1955) for dinitrophenol contractures).

V. 3. *Optical changes during a twitch*

V. 3. 1. *Scattering of light*

The early decrease in the amount of light diffracted by the striations, discovered by D. K. HILL (1949), was mentioned on p. 300. This early change is soon masked by a much larger effect, which appears to be a decrease in the amount of light scattered by the muscle, and which

follows roughly the time course of the tension (SCHAEFER and GÖPFERT, 1937; D. K. HILL, 1949, 1953). This scattering occurs almost entirely in the direction at right angles to the long axis of the fibres, and must therefore be due to longitudinal elements of the muscle structure. These are presumably (*a*) the outlines of the fibres themselves, and (*b*) the threads of sarcoplasm that lie between the groups of fibrils. If we set aside the possibility that the change depends on an alteration in the shape of the fibres, then the decrease in scattering is probably due to a decrease in the difference of refractive index between sarcoplasm and fibrils. In isolated fibres under the interference microscope, it is quite clear that the sarcoplasm has a higher refractive index than any part of the fibrils (A. F. HUXLEY and NIEDERGERKE, 1954), so that a decrease in the refractive index difference could indicate either a transfer of dissolved substances from sarcoplasm to fibrils, or of water in the opposite direction; the second seems a more likely possibility. There is no basis at present for estimating the magnitude of the shift.

V. 3. 2. *Decrease of birefringence*

It is well established that the strength of the birefringence of striated muscle falls during an isometric twitch (VON MURALT, 1932; BOZLER and COTTRELL, 1937), the amount of the fall being a maximum (about 30 per cent) if the muscle is near its natural length. This effect might also be accounted for by the shift of water from fibrils to sarcoplasm which was suggested in the last section as an explanation of the decrease in scattering. This could act in two ways: first, by concentrating the material which lies between the filaments in the fibrils, so reducing the refractive index difference which causes the form component of the birefringence, and second, by reducing any contribution, analogous to form birefringence, which may be made directly by the refractive index difference between the threads of sarcoplasm and the fibrils. If the movement of water went so far that the refractive index difference was actually reversed in either of these cases, then the form birefringence would increase again (since its strength is roughly proportional to the square of the refractive index difference); this is a possible explanation for the double-humped curves that were obtained by VON MURALT.

V. 4. *Tetanus tension*

V. 4. 1. *Absolute values*

The absolute values for the tetanus tension per unit cross-sectional area that are to be found in the literature for vertebrate muscle vary over a wide range. Frog muscle at 0°C appears to give from 1·5 to 2 kg/cm^2 (HILL, 1938; HAJDU, 1951); at room temperature, HAJDU finds 2·5 kg/cm^2, while RAMSEY and STREET (1940) obtained 3·5 kg/cm^2

from isolated fibres, which would correspond to about 3 kg/cm² in whole muscle when allowance is made for intercellular space. Larger values are generally quoted for mammalian muscle (e.g. 5 kg/cm² for rabbit muscle, WEBER, 1955, p. 273) but RITCHIE (1954) found only 1·5 kg/cm² in rat diaphragm strips at 37°C. It would be valuable to know whether these differences are real, or whether they are the result of some accidental difference in technique or in the arrangement of the fibres; if they are real, the question arises whether it is the number of active sites that varies, or the tension developed per site: the tension

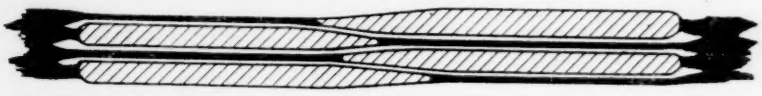

Fig. 12. Arrangement of fibres in a muscle which might give a spuriously high value for the tension per unit area. Tendon, black; muscle fibres, shaded. The tension exerted at the tendons by the fibres shown is four times the tension generated by any one fibre, while the cross-sectional area of muscle fibre at any point is only twice that of the cylindrical part of a single fibre.

per site is clearly important in developing theories of contraction (cf. p. 296). A point which does not appear to have been checked in all cases is whether the fibres of the muscle run from end to end. Even if the fibres are parallel, it would be possible for an arrangement such as is shown in Fig. 12 to produce a larger tension per unit area of the whole muscle than exists within the fibres themselves. The great majority of the fibres of the frog sartorius certainly run from end to end, but it is well known that in the longer muscles of vertebrates, a large number of fibres terminate with pointed ends within the muscle (ROLLETT, 1856; KRAUSE, 1869, pp. 2–6; ADRIAN, 1925).

A. F. HUXLEY and NIEDERGERKE (1954) pointed out that, if the tension in each filament is the sum of effects generated at a number of points in each zone of overlap between actin and myosin, then, other things being equal, a fibre with broad sarcomeres, and therefore a longer zone of overlap, would be expected to produce more tension than one with narrow sarcomeres. This would be compensated by slower contraction, since the number of contracting zones in series (double the number of sarcomeres) would be reduced in proportion. JASPER and PEZARD (1934) showed, in a comparison between different muscles of the same animal (crabs and lobsters), that speed goes with narrowness of striation, and BRENNER (1939) showed the same in a comparison between the three pairs of legs of *Dytiscus*, but I do not know of any data on absolute tension in these or similar cases. There is no comparable variation between different striated muscles of vertebrates; the striation spacing at rest length appears to be remarkably uniform at about 2·5 μ.

There does not seem to be any explanation at present for the variation of tetanic tension with temperature, nor for the failure of tension to be maintained in a constant current contracture.

V. 4. 2. *The limit to shortening in a tetanus*

The fall of active tension in a tetanus as a muscle is extended beyond its unloaded length finds a ready (though by no means proved) explanation in the decrease of the width of each zone of overlap between actin and myosin filaments, and consequent decrease in the number of sites at which tension can be developed (A. F. HUXLEY and NIEDERGERKE, 1954; this assumes that the sites produce tension in parallel). There is no correspondingly simple explanation for the fall of tension with shortening below the unloaded length. This takes the muscle into the range of lengths where the actin filaments must be shortened (perhaps crumpled at their ends); the force required to produce this deformation might be directly reducing the tension which appears at the ends of the muscle. In a tetanus which is not unduly prolonged, the extreme shortening brings the muscle just about to the point where the myosin filaments come into contact with the neighbouring *Z* lines; further shortening only occurs (at any rate in frog muscle) if the muscle is brought into the "delta state" (RAMSEY and STREET, 1940), which involves irreversible changes in the muscle. Again, it is conceivable that it is the stiffness of the myosin filaments that normally prevents further shortening. Alternatively, it may be that the degree of activation is progressively reduced as the muscle shortens; this is suggested by the experiment of KATZ (1939, fig. 6) in which he allowed a tetanized frog sartorius to shorten considerably and develop tension; he then applied a load greater than it could hold but less than the isometric tension the muscle could produce at its rest length. This load stretched the muscle beyond the point where the isometric tension should have been enough to hold it, and the muscle then shortened again to this point. This experiment suggests that the degree of activation was temporarily reduced, but there is no evidence whether this was a consequence of the shortening as such, or of the rapid stretch.

V. 5. *Distribution of other substances*

V. 5. 1. *Sarcoplasmic proteins*

It was mentioned above (p. 306) that the sarcoplasm has a higher refractive index than even the *A* bands of the fibrils, at any rate in isolated fibres from the frog. One would naturally expect the reverse, since refractive index is probably a good measure of the total concentration of solid and dissolved matter present, and the fibrils contain a large amount of actin and myosin in the form of filaments which are

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absent from the sarcoplasm. The *I* bands probably contain some other material as well as actin, since the refractive index difference between *A* and *I* suggests that the total concentration in *I* is about $\frac{3}{4}$ that in *A* (A. F. HUXLEY and NIEDERGERKE, unpublished), while this ratio should be about $\frac{1}{4}$ if the *I* bands contained only actin.

Presumably either the filaments are very highly hydrated, or else the soluble proteins of the sarcoplasm are at least partially excluded from the spaces between the filaments. The second of these explanations is suggested by the observation of WEBER (1934) that a dried "myosin" thread placed in a 15 per cent myogen solution would take up five or more times its volume of water without any change of dry weight, i.e. none of the myogen entered the thread. To explain this, WEBER postulated that the micelles composing the thread must be small enough, and therefore numerous enough, to leave spaces no larger than 90 Å (based on a molecular weight of 81,000 for the main component of the myogen, with an allowance for a shell of bound water). On a simple calculation, the spaces between the filaments of fresh muscle come out to be much larger than this. In the *A* band, where both sets of filaments are present, the X-ray data of H. E. HUXLEY (1952; HANSON and H. E. HUXLEY, 1955) give the distance between centres of a myosin filament and each of its neighbouring actin filaments as 250 Å; under the electron microscope the diameters of these filaments are 110 and 40 Å respectively (H. E. HUXLEY, 1953a), so that the gap is 175 Å. For this figure to be reduced to 90 Å by hydration, the filaments would need to be increased to more than twice the diameter found with the electron microscope, and even then the situation would not be explained since the gaps are very much wider in the *H* region and in the *I* band, and they also increase when the muscle is allowed to shorten; also, such a degree of hydration would probably be too high to be consistent with the strength of the form component of the birefringence, unless perhaps the filaments are tubular as is suggested in some electron microscope photographs (H. E. HUXLEY, 1953a; HODGE, 1955). An alternative possibility is of course that the fibrils are separated from the sarcoplasm by a membrane which is impermeable to the soluble proteins, but no such structure has yet been reported from electron microscope observations; or again, "long-range forces" may be involved. The situation is altogether puzzling and deserves further investigation.

V. 5. 2. *Ultra-violet absorbing material*

The well-known measurements of CASPERSSON and THORELL (1942) show nearly all of the material with an absorption spectrum resembling that of the purines to be localized in the *I* bands. They state (without evidence) that 95 per cent of the material with this absorption consists

of the adenosine phosphates; if this is correct, their observations must mean that the concentration of ATP in free solution in the muscle fibre is very much less than the average concentration obtained by dividing the fibre volume into the total amount of ATP present. This is clearly a point of great importance for interpreting the role of ATP in contraction; it appears to be commonly assumed that all the ATP is present in free solution.

There seem to be two loopholes in CASPERSSON and THORELL's work. The first is that their measurements were made on fixed fibres, and there was no actual check that some ultra-violet absorbing material did not escape from the fibres on fixation, though their photographs certainly show that the appearance of the fibres was not noticeably changed. The second is that figures for the nucleic acid content of muscle (DAVIDSON, 1947; LESLIE, 1955) suggest that ATP may form a much smaller proportion of the absorbing material than is assumed by CASPERSSON and THORELL. The location of the ATP in the living fibre is of such theoretical importance that a check of these points might be worth while.

It is interesting that some of CASPERSSON and THORELL's photographs show the ultra-violet absorbing band to be double, so that the distribution of absorbing material corresponds to the position of the *N* bands, which are probably formed by rows of regularly aligned granules (RETZIUS, 1890), together with other sarcoplasmic structures.

CASPERSSON and THORELL's experimental findings were confirmed by ENGSTRÖM (1944), who also showed that the ash produced by microincineration at 500°C was localized in the *I* bands. He believed this ash to consist of phosphates, and to be derived from the adenylic acids and from creatine phosphate, but he did not mention as a possible source either the nucleic acids or the phospholipids; the latter appear to be concentrated in the *I* band, at any rate in mammalian muscle (DEMPSEY *et al.*, 1946).

HOAGLAND, SHANK and LAVIN (1944) also found absorption at 2537 Å, again localized in the *I* bands (HOAGLAND, 1946). Their material was fixed in formalin and sectioned in paraffin, but there is no evidence whether some of the ultra-violet absorbing material had escaped.

VI. CONCLUSIONS

As a result of experimental work which has been carried out in the last three or four years, it is at last possible to give interpretations for the chief features of the striations of muscle. The following points can be regarded as very well supported, though perhaps not established beyond doubt in exactly the form stated.

(1) The *A* band does not change in width during stretch and moderate shortening.

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(2) The high refractive index and birefringence which distinguish the *A* band are due to rodlets of myosin.

(3) Filaments of actin run across the *I* bands and into the *A* bands as far as the boundary of the less dense *H* region, which represents the gap between adjacent sets of actin filaments.

(4) Except in extreme shortening, changes of muscle length take place not by stretching or shortening of either of these sets of filaments, but by their sliding past one another in each zone where they overlap.

(5) The dense *Z* line which bisects the *I* band is concerned with conveying the influence of the electrical changes in the fibre membrane which accompany excitation to activate the contractile myofibrils.

The point mentioned under (1) was foreshadowed by much evidence from nineteenth-century work, while evidence for (2) continued to accumulate up to about 1940. In each case, the old (and it now appears more correct) opinion was generally discarded, on evidence which now seems inadequate but which was supported at the time by theoretical considerations. This re-emphasizes the necessity for keeping experimental data clearly in view, whatever ideas about the mechanism of contraction may be current.

These interpretations of the striation pattern are of great interest from the point of view of the nature of the contraction process. In the first place, they are sufficiently definite and detailed to play their part, alongside the biochemical and biophysical information, in the formulation of hypotheses. In the second place, they are not easily reconciled with the current idea that contraction takes place by the folding of protein chains at a number of links in series; they therefore suggest new interpretations both of the contraction process itself and of many other phenomena in muscle. The greater part of this article is devoted to exploring some of the possibilities that are opened up in this way. A suggested mechanism of contraction, formulated with reference to the idea that shortening takes place by sliding, not folding, and to the main facts of the responses of glycerinated fibre preparations to ATP, is worked out in sufficient detail to show that it can account fairly well for the mechanical and thermal behaviour of muscle. Neither this however, nor the explanations that are tentatively proposed for various other phenomena, are at present beyond the stage of acting as working hypotheses.

Note added in proof

The experiments on *local activation of a muscle fibre*, apparently through the *Z* membrane (pp. 24–26), have been extended since this article was written (A. F. Huxley, *Proc. Physiol. Soc.* 2–3 November, 1956). The chief new findings are:

1. In crab muscle, the sensitive region is not opposite the *Z* lines but is near to each boundary between *A* and *I*. When the membrane

is depolarized over such a point, only the adjacent half-*I*-band (from *Z* to *A*-*I* boundary) shortens.

2. The experiments on frog muscle were repeated under the interference microscope, when the *Z* line is clearly seen. It was confirmed that the sensitive region is opposite the *Z* line, and the contraction always involved both halves of the *I* band equally, even when a weak contraction was evoked by placing one edge of the pipette over the *Z* line.

3. The extent of the inward spread of the contraction in frog fibres was found to be graded according to the strength of the applied potential even when the fibre as a whole was able to respond to an ordinary stimulus with an all-or-none twitch and propagated action potential.

4. In frog fibres, the sensitivity to depolarization does not extend continuously along the line of contact between the *Z* membrane and the sarcolemma, but is restricted to spots whose separation along the perimeter of the fibre is of the order of 5 μ.

Most of the conclusions reached on pp. 27–31 are strengthened by these results as far as the striated muscle of vertebrates is concerned, but clearly some structure other than the *Z* membrane is involved in arthropods. In the latter case, the *Z* lines must have some other function (cf. p. 30), and it is likely that the *Z* lines of vertebrate muscle perform this function, whatever it may be, as well as being involved in the inward spread of activation.

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CHLOROPLAST STRUCTURE AND ENERGY CONVERSION IN PHOTOSYNTHESIS

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The following abbreviations have been used in this article:
PGA, phosphoglyceric acid; ATP, adenosine triphosphate;
DPN^+, diphospho-pyridine nucleotide; TPN^+, triphospho-pyridine nucleotide;
DPNH, TPNH, the corresponding reduced forms; DNP dinitro-phenol;
FMN, flavin mononucleotide.

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CHLOROPLAST STRUCTURE AND ENERGY CONVERSION IN PHOTOSYNTHESIS

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I. Introduction

The mechanism of photosynthesis, the process by which green plants use light energy and water to convert carbon dioxide into organic carbon compounds, may be considered from two aspects. Firstly from the biochemical point of view, that is the study of intermediates and their interconversions, and secondly from the physico-chemical point of view, that is the study of the mechanism of conversion of radiant energy into chemical energy. The intermediates concerned with the evolution of oxygen are not yet known, but the manner in which the carbon of carbon dioxide is incorporated into organic compounds is now reasonably well understood. Such knowledge of the biochemistry of photosynthesis imposes certain limitations as to the possible photochemical mechanism involved. This article will briefly review what is known of the biochemistry of photosynthesis, and then discuss how far our knowledge of the photochemistry of chlorophyll is adequate for an understanding of the necessary driving force.

II. General Biochemical Mechanism of Photosynthesis

Calvin (1955) has reviewed the work of his group with radioactive carbon, and therefore only a brief discussion is given here. Radioactive carbon dioxide was supplied, and the compounds formed by the plant in the light and dark determined by the use of autoradiographs of paper chromatograms. With periods of exposure of several minutes many of the same compounds were labelled both in light and dark; as the period of exposure was shortened certain compounds were preferentially labelled in light. Both algae and higher plants have been used, and the percentage of the total activity fixed in individual compounds determined as a function of the period of illumination. If a single chain of reactions is postulated, it follows that for very short times of exposure much of the activity will be present in the first intermediate; after a longer time the second intermediate in the chain will have a larger fraction of the activity, and finally the products will slowly progressively accumulate activity. With the shortest periods practically possible (2 sec) the percentage activity in phosphoglyceric acid (PGA) was 77 per cent, and would extrapolate to the order of

100 per cent for zero time of illumination. CALVIN concluded from this that PGA is the first intermediate in carbon fixation. As the time of illumination is increased, the percentage activity in PGA falls and that of other compounds, e.g. sugar phosphates, increases. For still longer periods of illumination the secondarily labelled compounds begin to decrease in activity, and a third group of compounds (the final products) become labelled to an extent which progressively increases with the period of illumination.

The distribution of activity within the different carbon atoms of the molecules labelled was also investigated, and provided further evidence

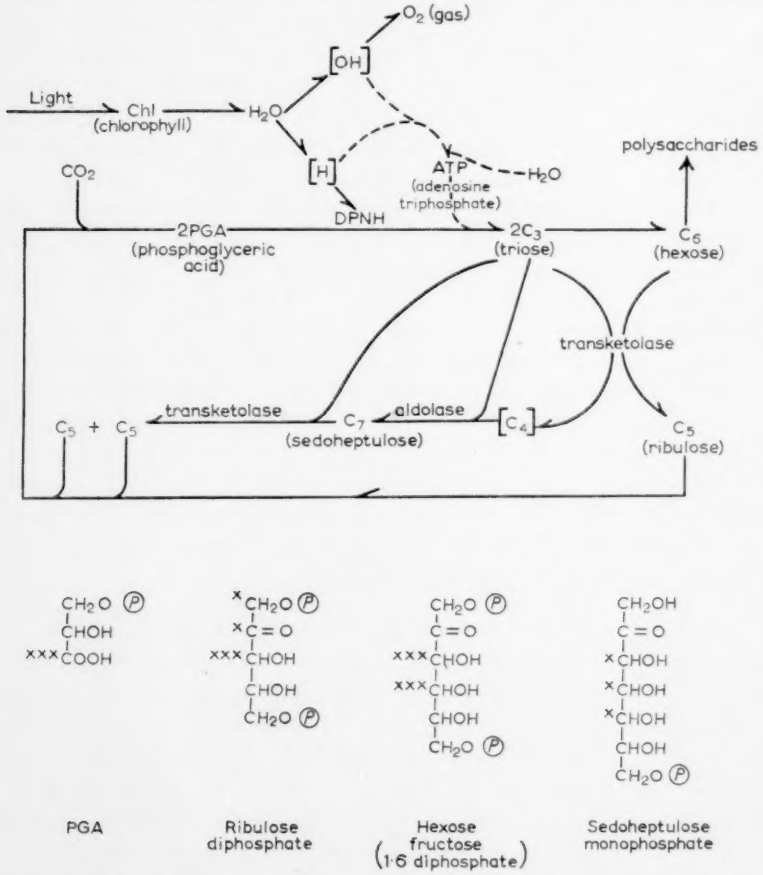

Fig. 1. The mechanism of carbon incorporation in photosynthesis (after CALVIN, 1955). The combination of a four-carbon fragment of the hexose with a triose will give the type of labelling observed in the seven-carbon compound sedoheptulose. The combination of a triose with sedoheptulose and a subsequent cleavage to two 5C compounds will produce one pentose labelled only in the 3C atom and one labelled in the 3, 4, and 5C atoms.

as to the sequence of their formation. The PGA is at first labelled exclusively in the 1C atom, the hexose equally in the third and fourth, the sedoheptulose phosphate in atoms three, four, and five, and the ribulose phosphate in atom three and to a lesser extent in four and five. Hence the ribulose could be formed from the sedoheptulose and the hexose from the triose. Fig. 1 illustrates a possible mechanism of photosynthesis which could account for this observed sequence of labelling. Kinetic evidence has been found in support of this mechanism. For example, reduction in the concentration of carbon dioxide results in a rapid decrease in radioactivity of ribulose, together with an increase in radioactivity of the three carbon compounds. Furthermore, the presence of the necessary enzymes has been demonstrated. Sonic extracts from *Chlorella* supplied with radioactive carbon dioxide and ribulose diphosphate form PGA in the dark (Quayle *et al.*, 1954). Spinach-leaf homogenates supplied with radioactive carbon dioxide, adenosine triphosphate (ATP), ribosephosphate, and reduced coenzyme (via. alcohol dehydrogenase or hydrogenase-coupled systems) will form labelled hexosephosphate in the dark (Racker, 1955). The mechanism illustrated in Fig. 1 suffices to interpret all the data so far obtained; furthermore it is consistent with contemporary work in other fields of biochemistry.

A consequence of this mechanism is that in the green plant for each molecule of carbon dioxide reduced to the level of carbohydrate, two molecules of reduced coenzyme (DPNH) and three molecules of ATP are required. ATP formation has been shown to accompany the oxidation of DPNH in both animal and plant mitochrondrial systems. If it arises in this or a related way in photosynthesis, the primary requirement to drive the photosynthetic cycle would be formation of DPNH. This could be formed ultimately from water by an oxidation-reduction reaction photosensitized by chlorophyll.

III. The Absorption and Transfer of Radiant Energy

The photosynthetic algae and higher plants contain within the photosynthetic centres, or chloroplasts, a number of pigments in addition to chlorophyll. It now seems certain that absorption by most of these pigments is effective in photosynthesis; the absorbed energy is transferred to chlorophyll *a*, the pigment common to all photosynthetic organisms (with the exception of the photosynthetic bacteria), and apparently most directly concerned with the photosynthetic reaction. Two types of evidence indicate that such transfer of energy does take place in the plant. Firstly fluorescence of one pigment may occur when the plant is illuminated with light of a wavelength which is almost exclusively absorbed by a second pigment. For example, the diatoms contain in addition to chlorophyll *a* a carotenoid pigment

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fucoxanthol. The quantum yield of chlorophyll fluorescence for light absorbed by fucoxanthol was found equal to that for light absorbed by chlorophyll (DUTTON, MANNING, and DUGGAR, 1943; WASSINK and KERSTEN, 1946). The same has been found true of the phycobilin pigments which occur in the red algae (HAXO and BLINKS, 1950). The second type of evidence in support of energy transfer is that the quantum efficiency of light absorbed by pigments other than chlorophyll

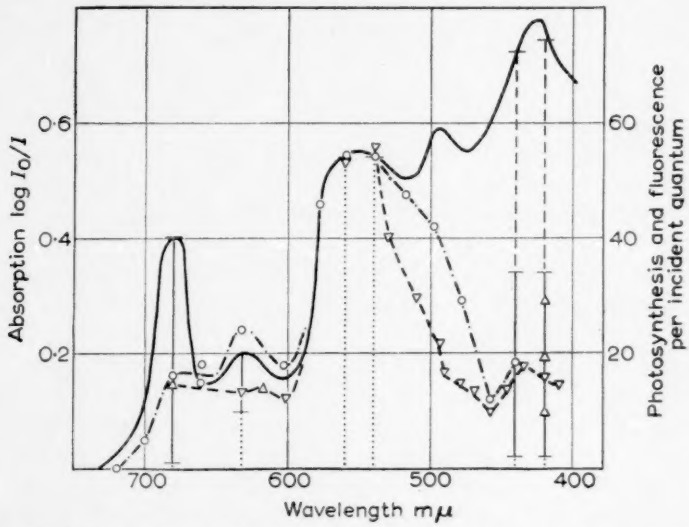

Fig. 2. The absorption and action spectra for photosynthesis and fluorescence in *Porphyridium cruentum* (after DUYSENS, 1952). The continuous line is the absorption spectrum; the broken line (· — ·) shows the photosynthesis, and the symbols (— —) the fluorescence per incident quantum as a function of wavelength. The vertical lines indicate the fractional absorption by individual pigments; — chlorophyll, ···· phycobilins, and — — — — carotenoids. The figure demonstrates that energy absorbed by chlorophyll or phycobilins results in photosynthesis and chlorophyll fluorescence.

is frequently as high as that of light absorbed by chlorophyll itself. In all cases so far examined there is an identity between the action spectra for photosynthesis and that for chlorophyll fluorescence (see Fig. 2). It is probable that chlorophyll *a* is the pigment most directly concerned in the photosynthetic process, the other pigments thus serving to extend the range of wavelengths which may be utilized by the plant.

The mechanism of transfer of excitation energy is presumed to be that of inductive resonance. The necessary condition for such transfer is that the fluorescence spectrum of the transferring molecule should overlap the absorption spectrum of the receiving molecule. The efficiency of transfer is high if the distance between the molecules is

less than 50 Å. Since there is an overlap of the fluorescence and absorption spectra of chlorophyll *a*, transfer of energy between chlorophyll *a* molecules themselves may be expected. The fluorescence yield of chlorophyll in the plant is about 1/10 or 1/100th that of chlorophyll in solution, indicating that the life of the excited molecule is such that about 100 transfers can occur in the plant (DUYSENS, 1952).

IV. The Photochemistry of Chlorophyll

The photochemistry of chlorophyll is most easily studied in homogeneous dilute solutions. Such studies indicate the existence of a ground state and two singlet excited states corresponding to the red and blue absorption bands. The spectrum and quantum yield for fluorescence of chlorophyll is the same whether excitation is by blue or red light. It follows that only one singlet state is the fluorescent state and the other state is shortlived (less than 10^{-10} sec), molecules transferring to the fluorescent state from this by a radiationless transition (internal conversion). The fluorescent or lowest excited state of chlorophyll has a life of the order of 10^{-8} sec and a maximum quantum efficiency of 0·25.

The existence of a long-lived metastable excited state (frequently assumed to be a triplet state) of chlorophyll has been postulated on the basis of kinetic and spectroscopic studies. Recently LIVINGSTON, PORTER, and WINDSOR (1954) demonstrated the existence of such a metastable state by observing the change in absorption during and consequent upon a brief intense flash of light. An intense flash was found in absence of oxygen to change reversibly about 90 per cent of chlorophyll *a* in dilute solution to a new form which showed a maximum in absorption at 475 mμ with a smaller shoulder at 540 mμ. The half-life of the new form was between 10^{-3} and 10^{-4} sec (LIVINGSTON, 1955). When illumination ceases chlorophyll should, therefore, emit light resulting from transfer from the metastable state to the ground state via the fluorescent state with the same wavelength distribution as fluorescence; in addition light emission could result from the return of the metastable state to the ground state directly, the wavelength maximum being longer than that for fluorescence. BECKER and KASHA (1955) have shown the existence of such an emission for chlorophyll *b* studied in rigid solvents at low temperatures, the maximum being at a wavelength of 865 mμ; unfortunately no emission was observed with chlorophyll *a*. From a study of porphyrin derivatives they found this type of emission to be a general property of this class of compound and to be greater if the contained metal ion was paramagnetic and of high atomic number. The metastable state for chlorophyll is considered to be some 33 K above the ground state and thus about 10·5 K below the fluorescent state.

The excitation energy of the lowest metastable (triplet) state is not

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sufficient according to FRANCK (1955) to permit transfer of hydrogen from the chlorophyll molecule even to such oxidants as molecular oxygen or quinone. FRANCK has suggested the existence of an excited triplet state resulting from the absorption of two quanta. The first quantum excites a chlorophyll molecule into the first excited state, it then transfers to the long-lived triplet state when it is again excited by transfer from an adjoining chlorophyll molecule in the first excited singlet state. With the estimated lifetime of the metastable state the rate of such photoactivation at low light intensities would be small; FRANCK points out that the probability of double excitation might be increased when the chlorophyll is closely packed on a suitable surface.

Thus there are three possible forms of excited molecule to initiate photochemical reactions. First, the singlet excited state with an energy per mole of 43 K, thought by some to be too shortlived to play any appreciable part in photochemistry; second, the metastable state with energy of 33 K; and last, an excited triplet state with energy possibly of 70 K.

In addition to the different forms of excited molecule, there are chemically different forms in the ground state which may play a part in photosynthesis. Chlorophyll in organic solution is reversibly bleached when illuminated in complete absence of oxygen. The absorption changes characteristic of the bleaching have been studied with chlorophyll in rigid solvents at the temperature of liquid nitrogen; they show a diminution of the red and blue peaks with enhancement of absorption in the far red (740 to 700 mμ) and in the yellow green 480 to 490 mμ (LINSCHITZ and RENNERT, 1952). A reversible oxidation of chlorophyll in methanol by ferric ions in absence of oxygen (RABINOWITCH and WEISS, 1937) results in a decrease in absorption between 460 and 550 mμ, but with no sharp band. The brown intermediate in the phase test, probably an oxidation product of chlorophyll, shows a decrease in absorption in the red and blue with new peaks at 470 and 540 mμ (WELLER, 1954).

KRASNOVSKII and VOINOVSKAYA (1952) have studied the reaction in absence of oxygen between chlorophyll dissolved in organic solvents with such acceptors as ascorbic acid, riboflavin, and DPNH. Chlorophyll *a* in toluene is photoreduced in presence of phenylhydrazine and then shows absorption bands at 518 and 585 mμ. The former band is attributed to the ion of the semiquinone form, the latter to the non-dissociated form.

With this knowledge of the properties of the chlorophyll molecule, one may speculate how far chlorophyll is itself chemically changed during photosynthesis. FRANCK (1955) has proposed a detailed mechanism in which it is supposed that the hydrogen atom bound to C_{10} of chlorophyll is labile. He suggests that the oxygen atom at C_9

in the keto form is hydrated, and that after excitation to the triplet state the hydrogen atom of C_{10} is donated to an acceptor, whilst one of the hydroxyl groups of C_9 is transferred to an enzyme from which oxygen is ultimately liberated. (See Fig. 3.) A double bond is formed between carbon atoms 9 and 10 and the chlorophyll reverts to the ground state in its enol form. Before considering in detail mechanisms

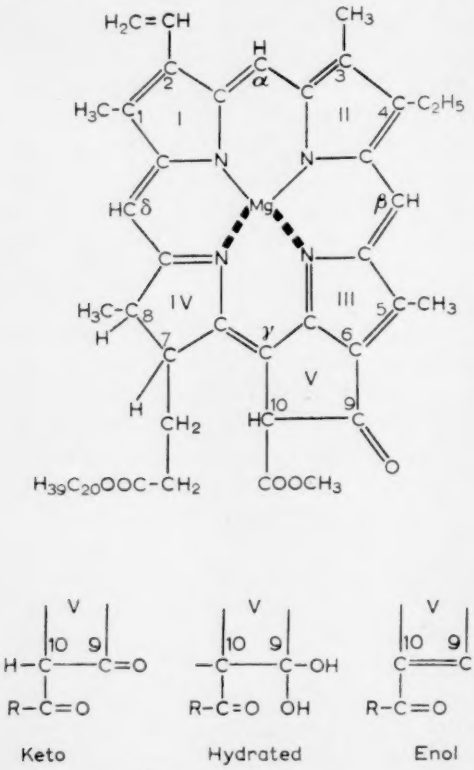

Fig. 3. *Above*: the chlorophyll A molecule. *Below*: configurations of the 9 and 10 carbon atoms.

in terms of reaction between a single chlorophyll molecule and water, it is necessary to consider the physical state of chlorophyll *in vivo*, and to have some knowledge of other molecules with which it is associated. For example, OSTER *et al.* (unpubl.) studied the effect of chemical bonding of dyes such as fluorescein and acriflavine upon their reaction with reducing agents such as ascorbic acid and glutathione. He showed that with certain dyes and weak reducing agents, photoreduction of the dye to the leuco form occurred only if the dye were bound to some polymeric substance, e.g. nucleic acid (compare OSTER, 1951).

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Fig. 4. Electron micrograph of a section of mesophyll cells from the leaf of *Nicotiana tobacum*. Magnification 18,600. Three chloroplasts are shown each with several grana. The smaller bodies with internal structure which lie between the chloroplasts are believed to be mitochondria. (From PALADE, 1953, by kind permission of the author.)

Fig. 5. Electron micrograph of a section of chloroplast from *Aspidistra elatior*. Magnification 43,000. The regions where the lamellae are closely packed are the grana; two adjacent grana may be mutually connected by several lamellae which continue through the intervening stroma region. With the use of a hand lens it may be seen that two opaque layers are joined at their ends showing the structure of the discs of the granum. (From STEINMANN and SJÖSTRAND, 1955, by kind permission of the authors.)

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Fig. 6. Electron micrograph of a section of a single cell of *Chlorella pyrenoidosa*. Magnification 57,000. The cup-shaped chloroplast is seen with lamellae extending throughout its length. In the bottom right of the cell the pyrenoid is visible. (From ALBERTSSON and LEYON, 1954, by kind permission of the authors.)

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V. Chloroplast Structure

The chlorophyll is concentrated in the plant cell in the chloroplast, a structure which shows considerable variation in external and internal morphology from plant to plant. During the past year the development of the Sjöstrand microtome for obtaining ultra thin sections has made possible electron micrographs of chloroplast structure of greater clarity than hitherto available. All chloroplasts examined show a laminated structure. In the algae e.g. *Chlorella* (Albertson and Leyon, 1954) the lamellae extend throughout the length of the chloroplast, whereas in the leaves of higher plants (with a few exceptions) the

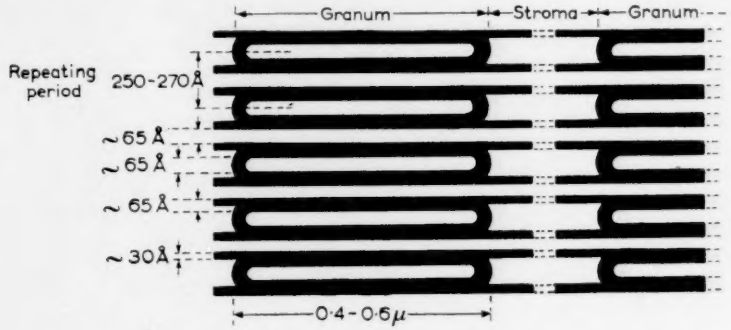

Fig. 7. Diagram of chloroplast structure according to Steinmann and Sjöstrand (1955).

lamellae are closely aligned in certain places along their length but are more loosely packed in between, e.g. Aspidistra (Steinmann and Sjöstrand, 1955). Figs. 5 and 6 illustrate these structures. The latter type of structure may be compared with the separation of highly pigmented regions ("grana") from regions with less pigment ("stroma") seen in the visible light and fluorescence microscopes. The chemical structure responsible for the differential absorption in the electron microscope after fixation with osmium tetroxide is not known. Steinmann and Sjöstrand find the lamella to consist of 2 membranes joined at their ends; each membrane is about 65 Å thick, and encloses a space of about the same size. From centre to centre of adjacent discs is about 250 Å (Fig. 7). These values are consistent with the view that the structure is that of a double protein layer with lipoid between. Some differences in detail of interpretation are suggested by Hodge, McLean, and Mercer (1955) on the basis of studies of the chloroplasts of *Zea mays*. It is tempting to relate this type of structure to the function of light absorption, and indeed a similar organization exists in the retinal rods; however, it is also present in mitochondria, which have no photochemical activity (Finean, Sjöstrand, and Steinmann,

1953). Frey-Wyssling (1955) suggests that in all these particles laminated structure may "facilitate exchange of energy and metabolites."

Wolken and Schwertz (1953) suggest that the chlorophyll molecules may form a monomolecular film separating the lipid from the protein. Chemically chlorophyll is well suited to this orientation since it has hydrophylic groups in the side chains attached to the porphyrin rings, and a lipophylic phytol chain. These authors calculated the average number of chlorophyll molecules in a chloroplast in the flagellates *Euglena* and *Poteriochromonas*, and then from the known dimensions of the molecule it was possible to calculate the area of surface required for a monomolecular film. It was of the same order as the area of interface available as calculated from the electron micrographs. It was thought that space would be available between the chlorophyll molecules in the film for carotenoid pigments containing hydrophylic groups, e.g. xanthophylls.

VI. The Chloroplast Reaction; Photolysis of Water

Hill (1939) first showed that chloroplasts isolated from the plant and, to a great degree regardless of their degree of fragmentation, liberate oxygen upon illumination in the presence of a suitable hydrogen acceptor. The general equation is

$$4H_2O + 2A \xrightarrow{h\nu} 2H_2A + O_2 + 2H_2O.$$

This now well-known reaction has been shown with quinone, ferric salts, and such dyestuffs as dichlorophenolindophenol as hydrogen acceptor. The chlorophyll acts solely as a photocatalyst. The reaction has been referred to as photolysis and is thought of as a "splitting" of water by light into an oxidized and reduced radical. The oxidized radical results in liberation of oxygen, the "hydrogen" in reduction of added acceptors. Wessels and Havinga (1952) have recently added a large number of substituted quinonoid compounds as reagents. Horwitz (1955) has shown that Janus green can act as a suitable acceptor, but that it is self inhibitory and not as good a reagent as ferric salts. Amongst biological compounds cytochrome C, DPN^+ and TPN^+ have been shown to accept hydrogen, the latter two only when the thermochemical back reoxidation reaction is minimized, by reducing the oxygen pressure and removing the reduced product by a suitable reaction system (Vishniac and Ochoa, 1952). Good and Hill (1955) added riboflavin and flavine adenine dinucleotide to the list of suitable reagents; this they demonstrated by continuously removing the reduced product by reaction with oxygen in presence of catalase and ethanol and determining the amount of reduction by the increased oxygen uptake. It has generally been considered that the acceptor for "hydrogen" from

the illuminated chloroplast need not be very specific. DAVENPORT, HILL, and WHATLEY (1952) have prepared from leaves a protein fraction which catalyses the reduction of methaemoglobin by illuminated chloroplasts. Activity has now been shown to be confined to a component in the protein fraction which runs electrophoretically as a single band. This substance also catalyses the reduction of cytochrome. In the latter case whole chloroplasts from chard were used which had little cytochrome C oxidase activity (DAVENPORT and HILL, 1955). Both components, methaemoglobin and cytochrome C, whose reduction is accelerated by the factor, are haem compounds.

There is no evidence to show that carbon dioxide is an essential reagent for Hill reaction activity as implied by WARBURG and KRIPPAHL (1955) who investigated the reduction of ferricyanide by whole *Chlorella* cells.* Many workers have shown photolysis in absence of any incorporation of radioactive carbon dioxide (BROWN and FRANCK, 1948; ARNON, ALLEN, and WHATLEY, 1954). There remains the possibility that carbon dioxide is necessary in catalytic amounts but if this is so, it must then be in a form not freely exchangeable with external CO_2; there is no evidence to support this.

Until recently the maximal rates of oxygen production per chlorophyll obtainable with chloroplast preparations at high light intensities ($Q_{O_2}{}^{chl}$) were considerably lower than the corresponding rates of photosynthesis. This may be due to the use of unsuitable reagents. An example of this is the use of quinone with *Chlorella* cells (Hill reaction). *Scenedesmus* appears to give higher rates with quinone than does *Chlorella* and the rate is apparently increased still further by addition of small amounts of 6·8-thioctic acid (BRADLEY and CALVIN, 1954). A very active isolated chloroplast system—perhaps the most active yet available—is that of DAVENPORT and HILL (1955 *l.c.*) using chard chloroplasts, the haem factor from pea, and cytochrome-C. $Q_{O_2}{}^{chl}$ of the order of 4000 have been obtained at 20°C, a value well within the range of values for photosynthesis.

VII. KINETICS OF THE CHLOROPLAST REACTION

The chloroplast reaction is inhibited by many substances which inhibit photosynthesis, e.g. hydroxylamine, o-phenanthroline, and phenylurethane indicating that there are reaction steps common to the two systems. Other substances markedly inhibit photosynthesis, but

* The author regrets that the term "Hill" reaction has been widely used to refer to oxygen production by whole algal cells in the presence of quinone or ferricyanide. The use of whole cells allows the possibility of oxygen evolution by normal photosynthesis, the added oxidant resulting in oxidation of carbon compounds to carbon dioxide in a dark reaction, which carbon dioxide is converted to oxygen in light. To avoid confusion the term "chloroplast" reaction is used in this article to refer to the photolytic production of molecular oxygen catalysed by isolated chloroplasts.

inhibit the chloroplast reaction only after prolonged exposure (e.g. cyanide) or not at all. Inhibitors of sulphydryl groups such as iodoacetamide and p-chloromercuribenzoate markedly inhibit photosynthesis but, as recently shown by FRASER (1953), WESSELS and HAVINGA (1953) and ARNON, ALLEN, and WHATLEY (1954) do not inhibit the photochemical reduction of quinone. Hence the primary photochemical reaction of photolysis does not require free SH groups for activity. On the other hand, the reduction of cytochrome C (or methaemoglobin) in the presence of the Hill and Davenport haem factor is inhibited by p-chloromercuribenzoate, and the inhibition is removed by addition of cysteine (DAVENPORT and HILL, 1955 *l.c.*). This presumably then is a more complex reaction than photolysis involving at least two steps of which the second depends on -SH groups; possibly the factor is itself unable to act catalytically without free -SH groups. The participation of -SH groups in chloroplast photochemistry cannot then be excluded except in the simpler photolytic reactions, e.g. quinone reduction.

The relationship between rate of oxygen production and light intensity has been compared for the Hill reaction and *Chlorella* photosynthesis. CLENDENNING and EHRMANTRAUT (1950) compared oxygen production from *Chlorella* in presence and absence of quinone as a function of light intensity. They found the same initial slope (i.e. quantum efficiency) and the same maximal rate, but a difference in rate for medium light intensities. However, their observed maximal rates were low (in the case of photosynthesis by a factor of at least 5) and it is possible both maximal rates were limited by the same limiting process. As stated previously, the maximal rate for the Hill reaction will be dependent on the oxidant used. DAVENPORT, HILL, and WHITTINGHAM (unpublished) have obtained light curves for pea chloroplasts in the presence of the factor catalysing the reduction of haem compounds and methaemoglobin, and it may be of interest to compare these with the light curve for photosynthesis of *Chlorella* for the same absorption of light energy by chlorophyll. In some experiments the same maximal rate for chloroplast reaction and photosynthesis was observed; but this was fortuitous, and in other experiments rates of chloroplast reaction were observed much higher than for the photosynthetic reaction ($Q_{O_2}{}^{chl}$ photosynthesis was 2500). In all our experiments, it was found that, despite differences in maximal rate and the fact that the two systems had the same rate at low light intensities, the chloroplast reaction had a lower rate than photosynthesis at intermediate light intensities. This may indicate a basic difference between the reactions immediately related to the photochemical process in isolated chloroplasts and in the cell.

Studies of the yield of oxygen by photosynthetic plants illuminated

with intermittent illumination have given evidence for the existence *in vivo* of several consecutive reaction steps following the photochemical reaction. When *Chlorella* is illuminated by light flashes of 10^{-5} sec duration the oxygen evolved per flash depends on the length of dark time between flashes, and with increase in dark time approaches asymptotically a limiting value (EMERSON and ARNOLD, 1932). Thus there is a limited amount of energy which can be utilized during a single flash; the maximal yield being one oxygen molecule for several (2–10) thousand molecules of chlorophyll. One interpretation of this is that associated with a large number of chlorophyll molecules is a single centre wherein the photochemical energy can be transformed into a chemical form. The dark time required between flashes will then be a measure of the working time of this centre. Recent experiments show that, if the duration of the flash is lengthened, a second limiting yield and dark time is observed. This suggests a sequential transfer of energy or reducing power along a chain of reactants.

Similar studies of the effect of intermittent illumination on the "Hill" reaction have also been made. EHRMANTRAUT and RABINOWITCH (1952) determined the yield per short (10^{-5} sec) flash for quinone reduction, and found the same dark reaction velocity constant for maximal yield per flash (10^{-2} sec) as for photosynthesis in *Chlorella*. ALLEN and FRANCK (1955) used longer flashes ($0{\cdot}5 \cdot 10^{-3}$ sec) and were able to measure oxygen production from a single flash with the quinone reaction but not with photosynthesis in *Scenedesmus* when the oxygen pressure was 10^{-6} mm Hg. A longer flash ($25 \cdot 10^{-3}$ sec) gave measurable oxygen production from both reactions. This difference may be related to the well-known induction effect present in photosynthesis but not apparent in the quinone reaction. A pair of short flashes even when separated by a dark period as long as 1 sec gave oxygen production in the quinone reaction in excess of twice that for a single flash, indicating the existence of some long-lived photoproducts.

BRADLEY and CALVIN (1955) showed that, with *Scenedesmus* illuminated by flashes of 10^{-4} sec duration, the dark time required to obtain maximal yield was a function of the quinone concentration. This indicates that in *Scenedesmus* (unlike *Chlorella*) the dark reaction is rate limited even by the highest concentrations of quinone which can be used without toxic effects ($3 \cdot 10^{-3}$ M). When 6·8 thioctic acid ($7 \cdot 10^{-4}$ M) was added in a concentration small compared with that of quinone the limitation was removed, and the dark time for maximal yield was reduced to the order of that found for *Chlorella*. As discussed in a later section this suggests a possible role for thioctic acid as an acceptor for the primary photochemical product.

HORWITZ (1954) investigated the rate of photosynthesis and of the quinone reaction in *Chlorella* as a function of light intensity in the

presence of H_2O and D_2O. At low light intensities substitution of D_2O for H_2O had little effect on the rate of photosynthesis; however, it did decrease the rate of the quinone reaction and to about the same extent as at higher light intensities. This suggests a possible difference in the way in which water reacts with the photochemical system. FRANCK (1955 *l.c.*) has suggested that in the quinone reaction the singlet state of chlorophyll may transfer hydrogen directly to quinone, recovering the hydrogen by reaction with water, whereas in photosynthesis chlorophyll in the metastable state is activated as a complex with the photosynthetic reactants.

VIII. REACTIONS OF CHLOROPLASTS OTHER THAN PHOTOLYSIS

VISHNIAC and OCHOA (1952 *l.c.*) showed that chloroplasts could in the light reduce DPN^+ and TPN^+; since that time it has been clear that in presence of a suitable enzyme system the reoxidation of this coenzyme by oxygen could be accompanied by phosphorylation. This type of oxidative phosphorylation, normally mediated by the cytochrome system, is dependent on the presence of oxygen or oxidized cytochrome, is inhibited by cyanide and uncoupled by DNP and methylene blue, and can be supplied with reduced coenzyme either in dark by addition of such acids as citric or malic or in the light in presence of chloroplasts.

More recently there have been two independent observations of a light-induced phosphorylation in plastid preparations which appears to differ from oxidative phosphorylation in at least some respects. FRENKEL (1954) showed that illumination of *Rhodospirillum* fragments in the absence of oxygen resulted in phosphorylation of added ADP; further, this reaction was not inhibited by DNP (10^{-4} M) or by iodoacetamide (10^{-3} M) but was inhibited rather weakly by cyanide (10^{-3} M). WHATLEY, ALLEN, and ARNON (1955) found with "whole" chloroplast preparations from chard a light-induced phosphorylation which was inhibited by oxygen; it was stimulated by addition of vitamin K, ascorbic acid, FMN and Mg^{++}, but not by addition of DPNH or TPNH. Furthermore, addition of tricarboxylic acid cycle substrates in the dark did not result in phosphorylation. WHATLEY *et al.* (*l.c.*) were thus led to describe this reaction as "photophosphorylation." They were able to prepare from the same leaves a particulate fraction of smaller average size which did not show photophosphorylation in light but was able to phosphorylate in dark when supplied with tricarboxylic acids. This dark phosphorylation was dependent on oxygen and inhibited by the usual inhibitors for oxidative phosphorylation. OHMURA repeated the work of WHATLEY *et al.*, and at first (1955a) obtained preparations of chloroplast fragments which gave oxidative phosphorylation in the dark when supplied with malic or citric acids but did not photophosphorylate. In later work he isolated the particles

in presence of ascorbic acid (1955b) and then obtained a preparation of larger particles ("whole" chloroplasts) which showed "photophosphorylation" and no oxidative phosphorylation, and another of smaller particles which in light showed photophosphorylation and in dark oxidative phosphorylation. He suggested that, since the same particles now exhibit both types of phosphorylation it is probable that photophosphorylation is simply the addition of photolysis and oxidative phosphorylation. However, no evidence was presented to show that the preparation of smaller particles was homogeneous. The lack of dark oxidative phosphorylation by whole chloroplasts might be attributed to inaccessibility of the reaction system (compare the absence of cytochrome oxidase activity); the nonrequirement for oxygen of photophosphorylation suggests that the oxidized radical resulting from photolysis ("OH") can terminate the oxidative chain, thus resulting in phosphorylation in absence of oxygen. Whether the OH radical reacts directly with a cytochrome will be discussed later.

M. B. Allen *et al.* (1955) showed that whole chloroplasts would fix $C^{14}O_2$ in the light. The products of this fixation have not yet been studied in such detail as have those for photosynthesis but it has been shown that radioactivity appears in sucrose and starch. Carbon-dioxide fixation and phosphorylation appear to be competitive processes, the competition presumably being for the primary reducing agent produced from photolysis. In agreement with this view the reactions can be shown to be differentially inhibited.

Thus the particulate preparations obtained from leaves are able to catalyse the photochemical production of both reducing power and phosphate esters. These are the two requirements to drive the biochemical mechanism proposed by Calvin and discussed at the beginning of this article. Thus the chloroplasts probably contain the whole of the photochemical (and perhaps also of the thermochemical) reaction mechanism of photosynthesis.

IX. Relation of Chloroplast Structure to Activity

Unfortunately there has been little attempt to examine with the electron microscope the structure of particulate preparations used for biochemical observations on chloroplasts. Palade's study of the leaf of the higher plant (1953) shows the presence of smaller particles in addition to the chloroplasts which he calls mitochondria (see Fig. 4). The degree of heterogeneity of particles within chloroplast preparations is frequently not known; neither is the degree of localization of enzyme systems within them. De Duve (1955) has made such a study with particulate preparations from animal tissue, and has shown a considerable degree of spatial separation; for example, TPNH—cytochrome-C reductase has maximal activity in the mitochondrial fraction

with some in the microsome (or smaller particles) fraction, whereas DPNH—cytochrome-C reductase is maximal in the microsome with less activity in the mitochondria. Further the DPNH cyt. C reductase of the microsomes is inhibited by antimycin A but that in the mitochondria is not. In view of Jagendorf's findings of the great difference in chloroplast activity in oxidation of DPNH and TPNH (1955) an investigation of the distribution of activity between leaf particulate preparations of differing average size would be of interest.

Of the enzymic activities shown by the isolated chloroplasts some are shown only by so-called whole chloroplast preparations, such chloroplasts being obtained by mild grinding in isotonic sugar solutions. For example M. B. ALLEN *et al.* (1955 *l.c.*) found that whole chloroplasts will phosphorylate and fix radioactive carbon dioxide in light; whereas after the chloroplasts were swollen in water (which results in separation of the lamellae as shown by MERCER *et al.* (1955), the preparation retained the ability to phosphorylate in light but lost the ability to fix carbon dioxide. The latter activity could be restored by addition of certain cofactors together with the water in which swelling had taken place. This water extract was inactivated by heating. Hence there are differing degrees of ease of extraction or susceptibility to damage of the different enzymic constituents. The most stable system is that catalysing the photolysis of water. THOMAS, BLAAUW, and DUYSENS (1953) showed that spinach leaf chloroplast fragments no greater than 100 Å in diameter (i.e. the width of a single lamella) still retained some 50 per cent of the photolytic activity of the whole chloroplast. MILNER, KOENIG, and LAWRENCE (1950) had previously shown that some restoration of activity of preparations of small chloroplast fragments was obtained by addition of aggregating agents, e.g. salts of monovalent cations. They also showed that loss of lipid material resulted in loss of ability to photoreduce dichlorophenol-indophenol. MCCLENDON (1954) showed with red algae, in which damage of the chloroplasts is easily recognizable by leakage of the water-soluble phycobilin pigments, that only when these pigments are kept *in situ* by the use of high-molecular-weight polymers (carbowax) was activity as measured by photoreduction of ferricyanide preserved. The grana structure of the chloroplast is probably necessary for the photochemistry of photolysis and photosynthesis; the structure of the chloroplast as a whole is not necessary for photolysis but is for the complete photosynthetic mechanism.

X. THE CHLOROPLAST REACTION *in vivo*

It appears probable that, built into the laminated structure of the chloroplast, there are specific molecules that accept the excitation energy of the excited chlorophyll. BASSHAM and CALVIN (1955) have proposed that the absorption by chlorophyll of a quantum results in

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the raising of an electron to a conduction band in which it may migrate through the orientated system of chlorophyll molecules. The structure must contain some polar character, such as exists at a *p-n* junction, so that electrons diffuse in one direction and the corresponding positive charges in the other. The lipid present in the chloroplast is thought to play the role of an insulator whilst the carotenoid molecules with their conjugated character act as conductors of electrons. Thus a separation of charge is attained in light, electrons accumulating at one surface and positive charges ("holes") at another. The electrons react with a chemical substance to form a reducing agent which ultimately reduces carbon dioxide; the positive holes react with water to liberate oxygen. Such a picture eliminates some difficulties which arise if we attempt to postulate a mechanism of reaction between a single chlorophyll molecule and water in terms of electron transfer one at a time.

Two types of mechanism have been suggested for the formation of a reducing agent consequent upon the absorption of a light quantum of 30 to 40 K. Firstly, one may consider that for each quantum one electron or hydrogen atom is transferred to a compound of as low an oxidoreduction potential as thermodynamically practicable. Alternatively, one may suggest a stepwise mechanism by which one quantum transfers a number of electrons or hydrogen atoms to an intermediate from which they are subsequently transferred to substances of lower and lower potential. Ultimately, by either mechanism, a compound with a potential at least as low as that of reduced coenzyme (DPNH) must be produced.

Bassham and Calvin (*l.c.*) proposed a mechanism of the first type in which 6·8 thioctic acid plays the role of electron acceptor. This compound is known to be present in chloroplasts, and has an oxidation-reduction potential slightly nearer that of the hydrogen electrode than reduced coenzyme. Two experimental results have been quoted to support the role of thioctic acid. Firstly, that in light there is little incorporation of radioactive carbon dioxide into the tricarboxylic-acid cycle intermediates; this is thought to be due to reduction in light of thioctic acid, the oxidized form of which is an essential cofactor for pyruvic-acid oxidation. Secondly, as mentioned previously, the quinone reaction of *Scenedesmus* is stimulated by addition of thioctic acid. Both types of experiment may be interpreted in a different manner, and the evidence is not unequivocal.

The second type of mechanism, in which reducing power is developed stepwise, was first proposed by Hill (1939) and later amplified by Davenport and Hill (1952) and others. The primary photochemical reaction was postulated to result in the reduction of a substance of a potential relatively near that of oxygen. By subsequent dismutative reactions, reducing power was transferred to another substance of

lower potential, the energy for this being derived from the part which was reoxidized. The hydrogen acceptor in the reoxidation is probably the oxidized radical derived from photolysis. Perhaps it is only fortuitous, but there are compounds in the green plant whose potentials are suited to such a role. The series, oxygen, cytochrome F, cytochrome B6 and coenzyme represent a hydrogen transfer series of approximately equal energy steps of the same order as the energy associated with a quantum of red light. WESSELS (1954) has suggested that vitamin K might be the initial electron acceptor; in this case two electrons could be transferred as the result of the absorption of one quantum. If this type of mechanism were operative it should in principle be possible to isolate partial reactions for which the quantum yield in terms of oxygen production would be an integral multiple of that for photosynthesis.

WARBURG, KRIPPAHL, SCHRÖDER, and BUCHHOLZ (1954) interpret some of their observations upon photosynthesis of *Chlorella* in this way. They consider that the photochemical reaction takes place between intermediates for which one quantum is sufficient to liberate one molecule of oxygen, the intermediates being formed in part from the result of oxidation in the dark of part of the photochemical product. The energy liberated in the dark oxidation is coupled to the photochemical reaction making this thermodynamically possible with a single quantum.

KAMEN (1955) has proposed a type of mechanism which, although of the first type, utilizes one of the cytochromes as intermediate. He proposed that the energy of a quantum absorbed by chlorophyll is transferred to ferrocytochrome bound within the chlorophyll protein matrix. It then transfers electrons which reduce DPN^+ and reverts to the ferro form by reaction with water with the concomitant liberation of oxygen. The mechanism postulates that the absorption of a quantum results in a loosening of the bonding between the cytochrome and protein in such a way that firstly the cytochrome becomes sufficiently reducing to reduce DPN^+, and secondly that the cytochrome is able to oxidize water. Evidence in support of these postulates has still to be found.

XI. Spectral Changes in Cell Suspensions

We return now to the discussion as to the form of chlorophyll molecule active in photosynthesis. Changes in spectra during photosynthesis may be expected to arise from two causes. Firstly, due to changes in the state of oxidation-reduction of certain biological compounds whose spectrum depends on their state of oxidation, e.g. the cytochromes and, secondly, to changes associated with the primary photochemical reaction, possibly due to changes in chlorophyll absorption.

Differences in spectra have been measured with whole cell suspensions, either by comparing the absorption of an illuminated cell suspension with that of a dark (i.e. very weakly illuminated) control or by comparing the difference in absorption between two illuminated suspensions, one with the wavelength to be investigated and the other with a different wavelength for which the absorption change has been previously shown to be negligible. The latter technique eliminates scattering effects and any physical change due to light. With *Porphyridium cruentum*

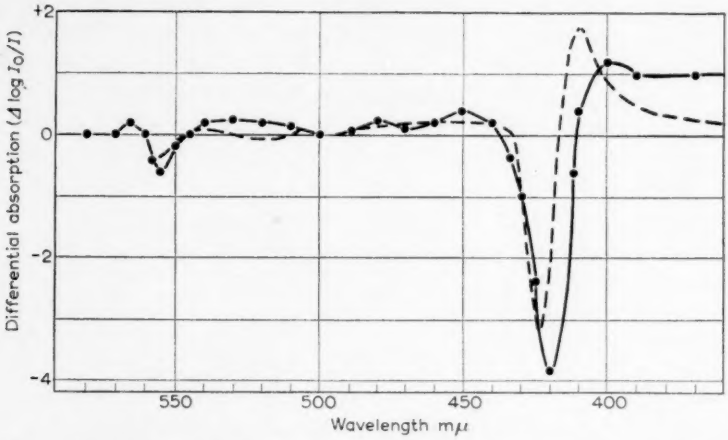

Fig. 8. The difference in absorption in light and dark of a cell suspension of *Porphyridium cruentum* plotted as a function of wavelength (continuous line). For comparison the difference spectrum for cytochrome F, a cytochrome peculiar to the chloroplast of green plants is shown (— — —). (After DUYSENS, 1955.)

DUYSENS (1955) found a difference spectrum which showed a marked increase in absorption at 420 mμ and a smaller increase at 550 mμ. This difference spectrum is closely similar to that of cytochrome F and indicates oxidation of a cytochrome in light. Previously LUNDEGÄRDH (1954) investigated a small-wavelength region between 550 and 570 mμ for *Chlorella*, and found a peak at 555 mμ which might also be due to oxidation of cytochrome F. DUYSENS (1954a) made observations with *Chlorella* over a wider range of wavelengths and showed the existence of maxima other than those which can be attributed to cytochrome; this will be discussed later. He also determined the difference spectrum for *Rhodospirillum rubrum* (DUYSENS, 1954b). This has been recently investigated in more detail by CHANCE and SMITH (1955). They determined the difference spectrum under a variety of conditions. Illumination of the bacteria under aerobic conditions produced only small effects, but under anaerobic conditions more marked changes were observed, similar to those resulting from addition of oxygen. Since *Rhodospirillum*

rubrum does not produce oxygen upon illumination, the effect of illumination cannot be attributed to production of oxygen. The observed difference spectrum indicates oxidation of cytochrome C2 (which corresponds to cytochrome F) and also of a cytochrome of the B type present in these organisms (VERNON and KAMEN, 1954; KAMEN and VERNON, 1955). Illumination in presence of sodium hydrosulphite, cyanide, or carbon monoxide, gave the same results as in their absence. CHANCE and SMITH postulate that one of the cytochromes acts as an oxidase which catalyses reaction between the oxidized radical produced photo-chemically from water and cytochrome F. This reaction is assumed not to be inhibited by cyanide or carbon monoxide. There was evidence that with illumination at certain wavelengths the oxidation of the cytochrome was sequential, the band at 430 mμ appearing first. OLSEN and CHANCE (unpublished) studied the difference spectrum of *Chromatium*, which is a strict photoanaerobe. Here there are no complications due to dark oxidation reactions, yet the difference spectrum is again consistent with an oxidation in light of haematin compounds. The oxidation of cytochromes as shown by the various difference spectra is evidence in favour of a mechanism of the chloroplast reaction of the first type, i.e. transfer to a powerful reducing agent in a single step.

DUYSENS (1954c) has also investigated the difference spectrum for *Chlorella* in the ultra-violet region. No sharp difference peaks were obtained, but the general increase in absorption is not inconsistent with the formation in light of DPNH.

In addition to the 555 mμ band observed in *Chlorella*, DUYSENS (1954a) observed an additional increase in absorption at 520 mμ and a decrease in absorption at 480 mμ. WITT (1955) observed the development of these absorption changes with duration of illumination. Both bands show the same time course, an initial phase upon which change in temperature has little effect and a second phase which is temperature dependent leading to the steady-state difference spectrum observed by DUYSENS. The fluorescence time course shows an exact parallelism. The changes in absorption are inhibited by inhibitors of photosynthesis, e.g. heating to 50°, phenylurethane. WITT suggests that the difference in temperature sensitivity of the two phases indicates that the change responsible for the primary phase is purely photochemical (perhaps due to chlorophyll) whereas the secondary phase is in part thermochemical. Changes in absorption at these wavelengths cannot be attributed, as yet, to any known metabolites. It is tempting to assume that they are characteristic of some form of chlorophyll. For this reason attempts have been made to determine whether there is any change in absorption consequent upon illumination in the red region, 680 mμ. Any such change appears to be of a smaller order of magnitude and is not yet

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fully established. Thus an understanding of the mechanism responsible for the changes at 480 and 520 mμ awaits further information.

XII. Chemiluminescence of Chlorophyll *in vivo*

Strehler and Arnold (1951) observed that, immediately after illumination ceases, green plants emit light having the same wavelength distribution as chlorophyll fluorescence (Arnold and Davidson, 1954). Chloroplast suspensions show a similar effect (Strehler, 1951). The emitted light has an intensity about 1/1,000,000 that of the exciting light. It is saturated by exciting intensities of the same order as those which saturate photosynthesis; it is inhibited by compounds which inhibit photosynthesis. It cannot, therefore, be emission from an excited state formed directly by light absorption, but must be associated with enzymic reactions. *Chlorella* cells show a time course for development of luminescence which is biphasic, whereas chloroplasts (without added hydrogen acceptors) show a progressive increase of luminescence with time of illumination up to 30 min. The decay of the luminescence with time is complex. After a brief flash, the decay follows a second-order reaction form at 6°C; but at higher temperatures the order is more complex (1·6 at 16°C) approaching first order at 25°C. After a longer flash the decay has an order of reaction of about 1·6. These observations have been interpreted in terms of a fast process with a small temperature coefficient and a slower process with a higher temperature coefficient. The half-time for the fast partial reaction is of the order of 1/100th of a second. Arnold and his co-workers interpret the luminescence as due to reversal of some of the early reactions in photosynthesis in such a way that chlorophyll is excited to the singlet state chemically.

XIII. Summary

Chlorophyll, when present in the chloroplast, can photocatalyse an oxidation-reduction reaction between water and various substances. *In vitro* this can be demonstrated with not too finely fragmented chloroplast preparations by addition of such substances as quinone or cytochrome. *In vivo*, spectral evidence suggests that in those photosynthetic organisms so far examined cytochrome is oxidized and possibly DPN^+ reduced. This is in agreement with the mechanism discussed, in which the absorption of a quantum results in the transfer of an electron from an excited chlorophyll molecule to some intermediate acceptor (possibly thioctic acid). Part of the reduced product is reoxidized via cytochromes to give phosphorylated derivatives, e.g. ATP, the remainder together with ATP serving to reduce phosphoglyceric acid presumably via reduction of a coenzyme.

The manner in which excited chlorophyll molecules participate in photosynthesis is not yet clear. In organic solution a metastable state

has been observed with a half-life of 10^{-4} to 10^{-3} sec. When chlorophyll is *in situ* in the cell, many phenomena are observed indicating some intermediate with a half-life of 10^{-2} sec. For example, this period is the duration of the dark time for maximal yield of photosynthesis in brief intermittent flash illumination and of the Hill reaction under the same conditions; it is the dark decay time for the initial spectral change observed by WITT; and it is one of the decay times observed with luminescence *in vivo*. BRADLEY and CALVIN (*l.c.*) propose that it is the average lifetime of an exciton produced as the result of absorption of a quantum in the chlorophyll-containing structure of the chloroplast. At suitable sites the exciton gives rise to an electron and positive hole; these respectively initiate the carbon-reduction and oxygen-evolving reaction sequences which together constitute the photosynthetic mechanism.

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